The proton-conducting channel of chromaffin granule membrane adenosine triphosphatase

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I declare that the thesis presented here has been composed by myself and that all of the work presented here was carried out by the author, with the exception of the determination of F_1ATPase content of chromaffin granule membranes which was carried out by Dr. D.K. Apps.

R. Sutton

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Abbreviations

ADP	Adenosine diphosphate
ADPNP	Adenylyl-imidodiphosphate
Ala	Alanine
ANS	1-anilinonapthalene-8-sulphonic acid
Arg	Arginine
Asx	The sum of aspartate and asparagine
ATP	Adenosine triphosphate
ATPase	ATP phosphohydrolase (EC 3.6.1.3)
ATPγS	Adenosine-5'-o-(3-thiotriphosphate)
BSA	Bovine serum albumin
β-octyl.glu.	1-o-n-octyl-β-D-glucopyranoside
Cho	Cholate
CTAB	Cetyl triethyl ammonium bromide
CTP	Cytidine-5'-triphosphoric acid
Cys	Cysteine
Cyt	Cytochrome
$C_{12}E_8$	Octaethyleneglycol dodecylether
DCCD	Dicyclohexylcarbodiimide
DEAE	Diethylaminoethyl
DIDS	4,4'-diisothiocyano 2,2-stilbene disulphonate
DNS, Dansyl	5-dimethylaminonapthalene-1-sulphonyl
d.p.m.	Disintegrations per minute
DTT	Dithiothreitol
Δ pH	Transmembrane pH gradient
$\Delta\psi$	Transmembrane potential
EDTA	Ethylenediaminetetraacetic acid
FCCP	Carbonyl cyanide p-triflouromethoxyphenylhydrazone

F_1	Soluble fraction of proton translocating ATPase
F_o	Membrane bound fraction of proton translocating ATPase
F_oF_1	Proton translocating ATPase complex
Gly	Glycine
Glx	The sum of glutamate and glutamine
GTP	Gaunosine-5'-triphosphate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonate
His	Histidine
IEF	Isoelectric focussing
IgG	γ-immunoglobulin
Ile	Isoleucine
INT	2(4-iodophenyl-3(4-nitrophenyl)-phenyl tetrazolium chloride
Leu	Leucine
LPC, Lysol	Lysophosphatidyl choline
Lys	Lysine
Met	Methionine
Mol.wt.,M_r	Relative molecular mass
NAD^+,NADH	Nicotinamide adenine dinucleotide
NbfCl	4-chloro-7-nitrobenzofurazan
NCCD	N-(2,2,6,6,-tetramethylpiperidyl-1-oxyl)-N'(cyclohexyl) carbodiimide
NEM	N-ethyl maleimide
NMR	Nuclear magnetic resonance
NP	Nonidet P
P	Pellet
Phe	Phenylalanine
pI	Isoelectric point
PP_i	Pyrophosphate

r.p.m.	Revolutions per minute
SDS	Sodium dodecylsulphate
Ser	Serine
SITS	4-acetimido-4'-isothiocyano-stibene-2,2'-disulphonic acid
SN	supernatant
SR	Sarcoplasmic reticulum
S-13	5-chloro-3-tert-butyl-2'-chloro-4-nitrosalicylanilide
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetraethylmethylene diamine
Thr	Threonine
Tris	Tris-(hydroxymethyl)-aminomethane
Trp	Tryptophan
TTX	Triton-X-100
Tyr	Tyrosine
UTP	Uridine-5'-triphosphate
Val	Valine

ABSTRACT

Chromaffin granules contain a membrane bound ATPase (F_oF_1) which hydrolyses ATP to form transmembrane proton gradients. The proton gradients are used in the uptake of catecholamines into the chromaffin granule. A soluble moiety (F_1) of this ATPase has been isolated by Apps and Schatz (1979) and was shown to be similar to F_1 from mitochondria. The F_oF_1ATPase is inhibited by dicyclohexylcarbodiimide (DCCD) at low concentrations, whilst isolated F_1 is only slightly sensitive to the aforementioned inhibitor. A protein of low molecular weight, different from a similar protein found in mitochondria, as judged by gel electrophoresis has been shown to bind ^{14}C-DCCD. ATP enhances binding of DCCD which also leads to greater inhibition of the ATPase. This protein has been shown to be associated with F_1ATPase both by column chromatography and two dimensional electrophoresis, after solubilisation of chromaffin granule membranes in nonionic detergent. The protein has been purified from membranes, after an initial delipidation, by organic solvent extraction. Column chromatography and liquid phase partitioning yield a pure protein. Amino acid analysis of this protein shows it to be similar to other DCCD-reactive proteins. The N-terminus has been shown to be blocked. Each chromaffin granule contains approximately 100 chains of DCCD-reactive protein and 5-10 ATPase molecules. This gives a DCCD-reactive protein:ATPase stoichiometry of 10-20.

Apps, D.K. and Schatz, G. (1979) Eur. J. Biochem., 100, 411-419.

Chapter 1

Thesis Introduction

1.1 The Project

The aim of the project was to study the nature of inhibition of the chromaffin granule ATPase (adenosine triphosphatase E.C.3.6.1.3) by N,N'-dicyclohexylcarbodiimide (DCCD) and this review attempts to cover work related to this project. The review is in three main sections, a general review of chromaffin granules, a review of the energy dependent mechanisms involved in substrate transport, and a review of similar ATPases from other sources.

1.2 Physiology of the chromaffin cell

Much interest has been focussed on the chromaffin system. It has long been known (Douglas, 1968) that adrenaline and noradrenaline are released from the adrenal medulla in response to nervous stimulation. The site of release of catecholamines was found to be located in the adrenal medulla within cells known as chromaffin cells. As early as 1953, Blashko and Welch had shown that two pools of catecholamines existed within the chromaffin cell, a free amine pool, and a pool of catecholamines trapped within membrane bound organelles, known as chromaffin granules. The same authors proposed that a nervous impulse caused depolarisation of the cell membrane, leading to release of cytoplasmic amine, the cytoplasmic store being replenished by catecholamines from the chromaffin granule. Douglas and Poisner (1966) showed that stimulation of the nerve terminating at the chromaffin cell resulted in release of ATP and also demonstrated the presence of large amounts of ATP within the granule. Kirshner et al. (1966) showed that a protein present in chromaffin granules was released from chromaffin cells upon nervous stimulation. These results suggested that the chromaffin cell releases adrenaline into the blood by an undefined mechanism of granule extrusion from the cell. However Trifaro et al. (1967) showed that no phospholipid was released from the chromaffin

cell upon nervous stimulation and so an exocytotic process, similar to that proposed for nerve terminals, was proposed. Thus, a nerve stimulus lead to depolarisation of the chromaffin cell membrane, fusion of the granule with the cell membrane, and release of the granule contents into the blood. Benedeczky and Smith (1972) showed that chromaffin granules in the process of exocytosis, that is granules fusing with the plasma membrane, could be found in freeze etched electron micrographs of secreting cells.

Biochemical studies have attempted to elucidate the exact sequence of events which give rise to plasma membrane-vesicle fusion. One of the most important observations is vesicle to vesicle aggregation promoted by Ca^{2+} (Edwards et al., 1974). This, coupled with the knowledge that free intracellular Ca^{2+} was the promotor of synaptic vesicle membrane fusion, led workers, who had noted the action of Ca^{2+} in enhancing actomyosin complex formation, to seek the presence of myosin in the chromaffin granule membrane. Burridge and Phillips (1976) using gel electrophoretic methods and Creutz (1977) using labelled antibody techniques suggested that any myosin found in chromaffin granule membranes was probably due to contamination by connective tissue.

More recently work has centred on three major areas. Use of isotonic gradients has lead to the purification of plasma membranes, and by making inverted vesicles of plasma membranes it was possible to study the effect of these vesicles on isolated chromaffin granules (Konings and de Potter, 1981). Work has also been done using cultured chromaffin cells, either isolated from collagenase treated glands or from adrenal carcinomas. By treatment of the cells with high voltages it was possible to cause dielectric breakdown of the cell membrane and effects of ions, adenine nucleotides etc. could be studied intracellularly

by diffusion of these molecules through the cell membrane (Baker and Knight, 1980). Finally isolation of a vesicle fusogen, originally called synaptin, but later synexin, from the adrenal medulla has led several authors to suggest synexin mediated plasma membrane chromaffin granule fusion in the chromaffin cell (Bock and Helle, 1977, Creutz et al., 1978).

Aunis et al. (1979) using freeze fracture electron microscopy have shown that, just before chromaffin granule-plasma membrane fusion there are distinct bridges connecting the two membranes. These bridges were interpreted as being proteinaceous. Using plasma membrane vesicles, Konings and de Potter (1981) suggested that just Ca^{2+} was required to trigger vesicle plasma membrane fusion with subsequent release of chromaffin granule contents. This is supported by the work of Meyer and Burger (1979) who have isolated three proteins from the plasma membrane which bind to glutaraldehyde fixed chromaffin granule membranes. Baker and Knight (1978) have shown in isolated adrenal cells treated with high voltages, that ATP is required for exocytosis, as well as Ca^{2+}. Hong et al. (1981) have shown that synexin promotes vesicle-vesicle fusion when reconstituted with liposomes. Finally Zinder and Pollard (1980) have proposed a novel method for release of contents from chromaffin granules. After synexin-induced chromaffin granule-plasma membrane fusion, cytoplasmic ATP produces osmotic lysis of granules (due to proton translocation) using blood Cl^- as a counter ion (see later). However the energy requiring step is unlikely to be the release of chromaffin granule contents into the blood as lipid bilayers have the same intrinsic energy whether in a flat or vesicular form. The overall conclusion that can be drawn from the work summarised here is that although the general sequence of events in exocytosis is understood the precise mechanism is not yet well defined.

Constituent	Concentration calculated from (1)	Concentration calculated from (2)
Catecholamine	570 mM	550 mM
Adenine nucleotides	155 mM	122 mM
Protein	180 mg/ml	169 mg/ml
Ca^{2+}	20-30 mM	17 mM
Mg^{2+}	4-6 mM	5 mM
Ascorbate	12 mM	22 mM
pH	5.5	5.6

Table 1.1. Concentrations of various constituents of the chromaffin granule matrix

The concentrations have been calculated from (1) Phillips et al. 1977, Phillips and Apps, 1979 and (2) Njus et al. 1980, Sen and Sharp, 1980, assuming 80% of the chromaffin granule protein to be soluble.

1.3.1 The internal contents of the chromaffin granule

In intact chromaffin granules, the appearance under the electron microscope is that of an electron dense core surrounded by a membrane, probably a lipid bilayer (see Winkler and Westhead, 1980, for a review). The internal contents can be released by osmotic shock of the chromaffin granules, and can then be separated from the membranes by centrifugation. The membranes obtained by this method can be resealed to form vesicles known as 'ghosts'. These ghosts display many of the transport properties of intact granules and have been used extensively in the study of these phenomena. Electron micrographs of the ghosts reveals membrane vesicles from which the electron dense core is absent (Winkler et al., 1972). Measurement of the concentrations of various components of the supernatant and the internal volume of the chromaffin granules gives an estimate of the concentration of soluble components in the granule matrix. Table 1.1 presents some collected data on the soluble contents of the granule. It is interesting to note that the major difference between the two concentrations given here, that is the data of Njus et. al. (1980) and Phillips and Apps (1979) can be attributed to different estimates of the internal water space of the chromaffin granule that is 4.3 μl.mg protein^{-1} by Phillips and Apps and 4.5 μl.mg protein^{-1} used by Njus et al. (1980). Also noteworthy is that considerably higher values of the soluble content based upon 90% of the proteins being soluble, was used to calculate the concentrations displayed in Table 2 of Phillips and Apps (1979). This may be due to a different method of granule lysis having been employed. Finally the results of Njus et al. take no account of the catecholamines which remain bound to the membrane after lysis, the data being derived from Winkler (1976) and based upon total granule lysis.

Of the soluble proteins the most abundant is chromogranin A, a

highly acidic protein (see Chapter 7), molecular weight about 77,000, which comprises about 50% of the soluble protein. Recent NMR data presented by Sen and Sharp (1980) have suggested that this protein is glycosylated. This result is supported by earlier work (Smith and Winkler, 1967b) but disputed by Aunis et al. (1975). Amino acid analysis reveals a preponderance of aspartyl and glutamyl residues which probably derive from aspartate and glutamate, giving the protein its low isoelectric point (pI). The protein is probably important in maintaining the low internal pH of the granule matrix: the NMR results do not suggest any strong complex formation with catecholamines.

Of the other soluble proteins dopamine β-hydroxylase (dopamine β-monoxygenase EC 1.14.1.7.1) constitutes 5% (Hortnagl et al. 1974; Helle et al., 1978), and low molecular weight acidic proteins comprise the remainder. It has recently been shown that the chromaffin granule matrix contains enkephalins (Viveros et al. 1979). Dopamine β-hydroxylase is present in both membrane bound and soluble forms. It is the only protein involved with catecholamine biosynthesis located entirely inside the granule (Laduron et al., 1976). This finding suggests that in adrenaline secreting cells dopamine first enters the granule, is converted to noradrenaline and then leaves the granule for conversion to adrenaline by phenylethanolamine N-methyl transferase (EC 2.1.1.28) a cytoplasmic enzyme. It is then taken up into chromaffin cells prior to exocytosis.

Of the nonprotein constituents of the matrix the majority of the molecules are catecholamines of which adrenaline is the most abundant, in bovine cells forming 72% of the total (Eade, 1958). Noradrenaline comprises most of the rest, with dopamine constituting about 0.8% (Eade, 1958; Sen and Sharp, 1980). These figures probably represent the proportions of adrenaline and noradrenaline-containing

granules in the bovine chromaffin cells, as granules highly enriched in noradrenaline have recently been isolated (Terland et al., 1979). The proportion of cells secreting adrenaline and noradrenaline varies between species (Aberer et al., 1979). Of the nucleotides ATP is by far the most prevalent with ADP comprising most of the remainder. The question of whether this represents the ratios of the nucleotides found in the cytosol has not yet been thoroughly investigated. Divalent metal cations have been shown to be present in the granule matrix (Phillips et al., 1977) Ca^{2+} being present at about 30 mM and Mg^{2+} at about 5 mM.

1.3.2. The complex of internal contents of the chromaffin granule

Since the cytosol of the adrenal cell is isoosmotic with 0.25 M sucrose (Blashko and Welch, 1953) and the internal contents of the chromaffin granule have an osmotic potential of around 1 M, some method of storing the granule contents in a way that decreases the internal osmolarity is implied. Early work (Helle, 1973) showed that at $0^{o}C$ high molecular weight complexes containing lipoprotein and catecholamines were formed although the presence of lipoprotein has since been disputed (Winkler, 1976). Recent results have suggested the presence of freely mobile lipid within the matrix (Sen and Sharp, 1980). Both the pH and temperature used were unphysiological in the work of Helle. Perhaps the most general criticism applicable to the NMR studies is that none of the whole granule studies were performed at physiological temperatures, whilst most of the studies in artificial media simulating the granule matrix were generally not performed at physiological concentrations of the internal contents of the granule. The nature of the internal complex has been reviewed extensively (Njus and Radda, 1977; Phillips and Apps, 1979; Winkler and Westhead, 1980) so this section will contain a brief description of results.

Early determinations of the ratio of catecholamines:ATP as 4:1 within bovine granules (Blashko et al., 1956) led to a suggestion that a neutral catecholamine-nucleotide complex might be involved. At pH 7.4 ATP carries four negative charges, enough to act as a counterion for the catecholamines which have one positive charge, but at pH 5.6 the pH approaches the pKa of the γ-phosphate of ATP. Thus at intragranular pH the ATP cannot provide the total counterion balance for the catecholamines. Furthermore the ratio is different in granules from other species. The most widely used tool for the study of the internal complex of the matrix is NMR spectroscopy. The chromaffin granule exhibits a broad spectrum (indicative of a highly ordered system such as a folded protein) which can be attributed to the membrane, and a series of well defined peaks which can be assigned to the matrix components. The rotational correlation times (calculated from the line width) of the NMR spectra reveal little crystalline like interaction or high molecular weight complex formation within the granule matrix (Sharp and Richards, 1977a, b) and indicates that individual matrix components are essentially in free solution. Of some significance is the observation that the peaks corresponding to amino acid residues are very sharp indicating that chromogranin A is in an extended freely mobile form within the matrix, results confirmed using circular dichroism (Rosenheck and Schneider, 1973). Daniels et al. (1978) were able to extract quantitative estimates of the tumbling rates of individual molecules (equivalent to three dimensional movement) which indicated some slowing down of tumbling rates within the granule. This, coupled with studies on artificial mixtures of adrenaline and ATP has led to a proposed stacking of catechol and purine rings, based upon shifts in the assignable ^{13}C and ^{3}H peaks to indicate interaction between molecules (Granot and Rosenheck, 1978). These results have been

strengthened by the work of Tuck and Baker (1973), who used ^{31}P-NMR spectroscopy with artificial mixtures of catecholamines and nucleotides, and proposed a weak ionic interaction between the protonated amine and the negatively charged phosphonucleotide. The role of Ca^{2+} in promoting catecholamine-ATP interactions has been investigated by Granot and Rosenheck (1978) who suggested that divalent metal cations may promote catecholamine:nucleotide interactions, although the matrix concentrations of Ca^{2+} and Mg^{2+} are much lower than those of catecholamines. The overall view presented here for the composition of the matrix is that of a free solution in which the osmolarity is reduced by weak interactions between Ca^{2+}, ATP and catecholamines, with chromogranins having little effect, although a role has been proposed for chromogranins (Sharp and Sen, 1978).

1.4.1. The chromaffin granule membrane

Surrounding the matrix of the chromaffin granule is a limiting membrane, as shown by the electron microscopic studies of Benedeczky and Smith (1972). The role of the membrane in the function of chromaffin granules has been approached in two different ways: these being studies on the physical composition of the granule membrane, and of the transport phenomena exhibited by the membrane, both in intact granules and chromaffin granule ghosts.

1.4.2. The lipids of the chromaffin granule membrane

The lipids of the chromaffin granule membrane present two major points of interest, the cholesterol and lysophosphatidylcholine (LPC) contents. The lipid composition has been reviewed by Winkler (1976). In general the chromaffin granule membrane is similar in phospholipid composition to the membranes of adrenal microsomes except that the relative phosphatidylcholine content is much decreased (42% of the total

phospholipids in microsomes, 26% in chromaffin granules) as shown by the analysis of Blashko et al. (1967). In contrast the chromaffin granule contains 16.8% LPC whereas the microsomes contain only 2%. It is unlikely therefore that the LPC content derived in these early experiments was an artefact due to the preparation procedure of the lipids as a similar concentration would be expected to be seen in the microsomal fraction. The presence of LPC has been confirmed subsequently by Voyta et al. (1978) and de Oliveira Filgueiras et al. (1979) although its presence has been disputed by Arthur and Sheltawy (1980). Originally it was thought that LPC could be involved in membrane fusion during exocytosis as it had been shown to increase membrane fusion in hen erythrocytes (Ahkong et al., 1972). More recent evidence, however, de Oliveira Filgueiras et al. (1979) using LPC lipase, has shown that LPC is probably situated on the inner face of the bilayer, although 30% of the LPC remains shielded from LPC lipase.

Studies of the lipid ordering (Bashford et al., 1976; Marsh et al., 1976) using spin label probes indicated that the lipids were formed into a bilayer lipid leaflet, with very little structure being imposed by the protein content. The bilayer structure has been confirmed by ultra-structural studies using freeze fracture electron microscopy (Plattner et al., 1969). The ESR data indicated that lipid undergoes a phase transition at about 33^{o}C, becoming more mobile above this temperature. This has been shown to correlate with discontinuous enzyme activities of the ATPase and the NADH oxidoreductase, indicating close association of lipid with these enzymes (Buckland et al., 1981). As noted by Njus and Radda (1977) the content of cholesterol is about 30% of the total lipid (Dreyfus et al., 1977) and at about this concentration of cholesterol significant changes occur in the morphological state of artificial vesicles, their size decreasing as the cholesterol content increases.

In contrast to this role for cholesterol Winkler and Westhead (1980) suggest that the cholesterol functions, not in vesicle size definition, but as a bulk constitutent for LPC which would otherise disrupt the bilayer. The topography of cholesterol has not yet been investigated. Perhaps two final points of interest concerning the lipids needs to be mentioned: one is the high lipid:protein ratio when compared with other organelles or with plasma membranes. Also if one sums the LPC and phosphatidylcholine contents of the granule and compares it to microsomal or lysosomal data the phospholipid composition is very similar. This is consistent with a common biosynthetic origin for all three membranes.

1.4.3. Chromaffin granule membrane proteins

The membrane proteins comprise most of the enzymes associated with the chromaffin granule, as well as some other proteins the function of which is unknown. Perhaps the most prominent band on SDS polyacrylamide gels run in the presence of reducing agents (Winkler et al., 1970; Jockush et al., 1977; Abbs and Phillips, 1980) is about M_r = 75 000 (M_r is defined as relative molecular mass), and has been identified as the enzyme dopamine β-hydroxylase. It differs from the soluble form of the enzyme, both in Arrhenius effects (Aunis et al., 1977) and in charge shift electrophoresis (Skotland and Flatmark, 1979; Bjerrum et al., 1979). Use of impermeant probes such as lactoperoxidase and ^{125}I labelling or pronase treatment, suggests that dopamine β-hydroxylase is situated on the extracytoplasmic face of the membrane, confirming the immunocomplement experiments of Koenig et al. (1976). This explains the observation that dopamine β-hydroxylase activity was Mg^{2+} ATP stimulated (Kirschner, 1957) in intact granules (see later). Other authors claim to observe anti-dopamine β-hydroxylase antibody

binding in intact granules (Thomas et al., 1974), but it is not clear from the work of Thomas whether the granules were intact. More recently Zaremba and Hogue-Angelletti (1981) showed in intact granules that dopamine β-hydroxylase was susceptible to pronase treatment, although this result is in direct contrast to the observation of Abbs and Phillips (1980). Probably the extensive washing procedure adopted by Zaremba and Hogue-Ange letti rendered the granules very fragile and susceptible to lysis.

The other major band observed on SDS polyacrylamide gels is chromomembrin B, M_r = 23-27 000, comprises about 10% of the total protein and has recently been separated into three components (Abbs and Phillips, 1980; Apps et al., 1980c). This was shown to be a hydrophobic protein (Flatmark and Gronberg, 1981) and has recently been identified as cytochrome b_{561} the only cytochrome present in the chromaffin granule membrane (Apps et al., 1980c). This band was shown to be only partially digested by pronase (Abbs and Phillips, 1980) which indicates that the protein is only slightly exposed at the cytoplasmic membrane surface; it is much more susceptible in broken membranes, suggesting that it is a transmembrane protein. Earlier work on the purification of the cytochrome b_{561} by Silsand and Flatmark (1974) which suggested a molecular weight of 4380 for the protein has since been amended (Flatmark and Gronberg, 1981). At the moment the function of the cytochrome has not yet been elucidated, although as its midpoint redox potential lies between those of dopamine β-hydroxylase and NAD^+/NADH it may serve as a bridge between the two enzymes across the membrane, the NADH-ferricyanide oxidoreductase being situated on the cytoplasmic face of the granule membrane. However it has not been possible to implicate this cytochrome in catecholamine biosynthesis (Grouselle and Phillips, 1982).

Other defined bands are α-actinin and actin (Jockusch et al., 1977). Although the peak of the band identified as actin by these authors did not comigrate exactly with actin, chromaffin granules were labelled by fluorescein-conjugated anti-actin Ig G (Da Prada et al., 1977; Wilkins and Lin, 1981). Of the enzyme activities of the chromaffin granule membrane only the ATPase has been well characterised. Other proteins include a phosphatidylinositol kinase (Phillips, 1973) the active site of which is situated on the outside of the granule, and is inhibited by the sulphydryl reagent N-ethylmaleimide, and an NADH acceptor oxidoreductase activity also on the cytosolic face of the granule. In total, of the 60-70 bands observed by Abbs and Phillips only 7-10 have been identified. Whether some of the bands are due to contamination by other organelles is as yet unclear, but of the enzymes already mentioned, other subunits of the ATPase, transporters of Ca^{2+}, catecholamines, nucleotides, anions and the Ca^{2+}/Na^{+} exchange protein must contain polypeptides and have not been identified (Phillips and Apps, 1979; Phillips, 1981).

1.5 Transport activities of the chromaffin granule membrane

1.5.1. Catecholamine transport

Since in the chromaffin cell the cytoplasmic concentration of catecholamines is very low (probably = 20 μM), whilst within chromaffin granules the catecholamine concentration is very high, some method of accumulation of these biogenic amines into the granule is necessary. Initial work by Blashko et al. (1956) and Falck et al. (1956) indicated that binding of catecholamines to an intragranular complex could provide the driving force for catecholamine uptake. However Kirshner (1962) and Carlsson (1962) observed that addition of Mg ATP to the granules greatly stimulated catecholamine uptake. Kirshner also noted that uptake was

inhibited by the alkaloid reserpine (used clinically as a tranquilliser) and was stereo-specific for the natural, (-)-form of the catecholamine. These three observations have been sited as evidence in support of a carrier mechanism, but catecholamines have been observed to accumulate slowly in liposomes in response to an applied pH gradient (Nichols and Deamer, 1976). This presumably occurs in the same way as weak bases distribute according to transmembrane pH gradients, crossing the membrane in an uncharged form. This transport has also been shown to be reserpine sensitive (Njus et al., 1980).

More recently chromaffin granule 'ghosts' were shown to accumulate catecholamines in the presence of Mg ATP (Taugner, 1972; Phillips, 1974). Both authors proposed that this was due to a Mg^{2+} dependent catecholamine translocating ATPase in which ATP hydrolysis was directly coupled to catecholamine transport. However data presented by Phillips in which catecholamine transport but not the ATPase activity were inhibited by reserpine were difficult to explain on this model. Either two ATPases, or specific catecholamine anion interactions had to be suggested in order to explain these observations.

However in a series of articles (Bashford et al., 1975; 1976a, b; Casey et al., 1976) the Oxford group led by George Radda demonstrated that the effects of 'uncouplers' such as carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) could be ascribed to collapse of the proton gradient across the granule membrane. The model, based upon the chemiosmotic hypothesis of Mitchell (1973), was that the proton motive force across the membrane was the driving force for active transport phenomena exhibited by a variety of membrane systems. Radda's group argued that the ATPase pumped protons into the granule matrix and that proton gradients thus generated could be coupled to catecholamine transport. Evidence was presented for this theory using 1-anilonapthalene-8-sulphonic acid (ANS), whose fluorescence had been shown to

respond to energization of submitochondrial particles (Radda and Vanderkooj, 1972). This energising effect had been ascribed to a mixture of the charge and the pH gradient across the membrane. In chromaffin granules the nature of the fluorescence changes were similar to those in submitochondrial particles i.e. ANS fluorescence was increased in the presence of Mg ATP abolished by uncouplers and reduced by catecholamines.

As part of their hypothesis (Casey et al., 1977) the catecholamine transporter was an obligatory proton/catecholamine antiport. Phillips (1978) using chromaffin granule ghosts demonstrated that catecholamines could be accumulated against an applied pH gradient, whilst Njus and Radda (1978, 1979) showed that an applied potential could also drive catecholamine transport, albeit at a much lower rate. Phillips (1978) found it difficult to explain the effects of anions in his experiments, whilst the ratio of catecholamines inside to outside of the ghosts observed by Njus and Radda (1979) were very much lower than those in the experiments of Phillips. Several other workers developed the work of Phillips (Schuldiner et al., 1978; Ingebretsen and Flatmark, 1979) and subsequently proposed an electroneutral mechanism, based upon the observation that in chromaffin granule ghosts amine transport caused no change in the membrane potential, while the pH gradient was dissipated.

The concept of an electrogenic proton/catecholamine antiport was proposed by Johnson and Scarpa (1979). This was prompted by the observation that in chromaffin granules Mg ATP stimulated catecholamine transport without much affecting the pH gradient, the pH change being largely buffered by the internal contents of the granule. The membrane potential caused by Mg ATP increased dramatically (inside positive) probably due to proton entry. In a series of studies (Johnson et al. 1978; Johnson and Scarpa, 1979; Johnson et al. 1979) catecholamine

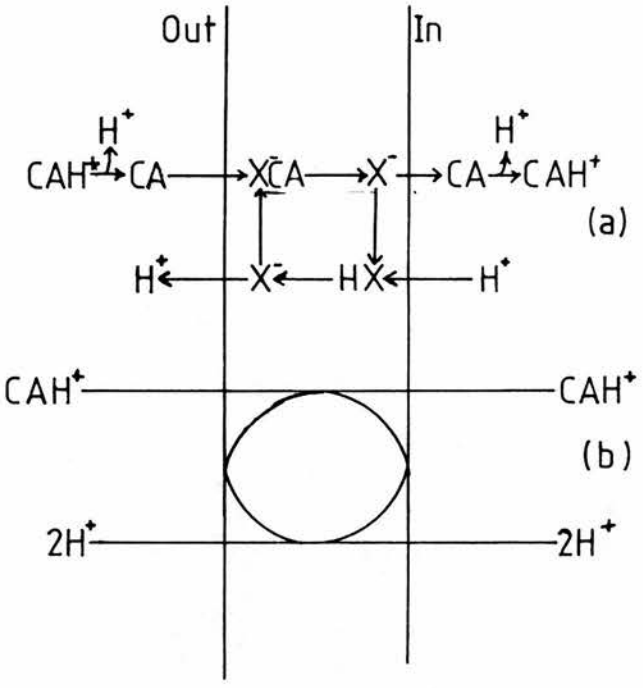


Figure 1.1. Catecholamine transport into chromaffin granules

Two models demonstrating alternative mechanisms by which catecholamines could be accumulated utilising a proton gradient, based upon (a) Johnson and Scarpa, 1979 and (b) Njus and Radda, 1979. CA = catecholamine, X = transport protein.

transport was shown to be electrogenic.

In the presence of ammonia (0-60 mM) the membrane pH gradient (Δ pH) could be collapsed. This is due to NH_4^+ distributing itself according to the transmembrane pH gradient and crossing the membrane as NH_3. The high concentration of external NH_4^+ required to collapse the pH gradient is presumably a result of the internal buffering capacity of the granules. The pH gradient could be measured in a similar way by ^{14}C-methylamine distribution. In a similar way SCN^- could be used to collapse the membrane potential ($\Delta\psi$) and both Δ pH and $\Delta\psi$ could be varied independently. They also proposed a model for catecholamine transport which is presented in Fig. 1.1(a). An alternative but related model which also accounts for the electrogenicity was proposed by Njus and coworkers and is presented in Fig. 1.1(b). Other groups have since presented evidence in favour of an electrically dissapative mechanism of catecholamine accumulation (Kanner et al., 1979, 1980; Apps et al., 1980(a)). Maron et al. (1979) and Isambert and Henry (1981) have reconstituted pH-dependent and ATP dependent catecholamine uptake in liposomes containing cholate solubilised chromaffin granule membranes.

More recent work has attempted to discern which of the two mechanisms presented in Fig. 1.1 drives catecholamine uptake in chromaffin granules. An interesting feature is that estimation of the proton/catecholamine stoichiometry limits either model to one form, and a stoichiometry of two protons/catecholamine has been found (Phillips and Apps, 1980). In a more recent work Johnson et al. (1981) emphasizes that either model could be correct based upon a thermodynamic analysis of the relationship between proton motive force and catecholamine transport. One way of distinguishing between the two mechanisms which basically only differ by which species of catecholamine, the cation or neutral amine is transported, is to look at the pH dependence of

catecholamine transport. Two attempts to resolve the situation by this method have been tried (Knoth et al., 1981; Scherman and Henry, 1981) and have yielded directly conflicting results, each favouring one of the models. The data presented by Knoth et al. (1981) shows only transport measured at three different pH, whilst the results of Scherman and Henry show a peak at pH 7.8 rather than the increase in predicted by these authors. This recent work will need to be completed in more depth, and it has been noted that the carrier may have different substrate affinities at different pHs (Knoth et al., 1981). At the present time the precise mechanism of proton/catecholamine antiport has yet to be defined.

1.5.2. Nucleotide transport

As mentioned earlier, nucleotides are also present within the chromaffin granule matrix in high concentrations (150 mM). Early work (Lagercrantz, 1970) showed that chromaffin granules were unable to synthesize ATP, the major chromaffin granule nucleotide. These results were confirmed by Winkler and coworkers (Peer et al., 1976) who demonstrated the most likely source of chromaffin granule ATP was the adrenal mitochondrion. ATP content may well be maintained by an active uptake process and Winklers group had shown that granule nucleotide content was maintained by the addition of Mg ATP to the medium. However it was not clear whether Mg ATP was maintaining ATP content by maintaining the catecholamine content with ATP simply acting as a counterion for the catecholamine.

Kostron et al. (1977) using ^{3}H adenosine and γ-^{32}P-ATP presented evidence to suggest that the uptake of nucleotides was carrier mediated in that uptake showed saturation kinetics, was inhibited by atractyloside, known to inhibit the mitochondrial nucleotide transporter (Klingenberg,

1980), and inhibited by reducing the temperature to 0°C. However since chromaffin granules contain high concentrations of ATP it was not clear whether ATP uptake was due to ATP exchange between the granule matrix and medium, the overall effect appearing as uptake as radioactivity was monitored. Uptake with ^{3}H-ATP was definitely shown to occur in chromaffin granule ghosts (Phillips and Morton, 1978) whilst inhibition by atractyloside suggested that nucleotide transport was not coupled to catecholamine uptake.

Two questions still remained; whether there was one carrier for all the nucleotides present in the granule matrix (see earlier) and whether nucleotide uptake was energy dependent. Even if the carrier was not a nucleotide/proton antiport, it may be expected that 'energising' the membrane (Bashford et al., 1975) would stimulate uptake as the potential applied across the membrane (inside positive) by ATP hydrolysis would be able to drive ATP uptake. This was shown to be the situation (Phillips and Morton, 1978) as addition of nigericin and potassium to bathing media of the ghosts did not affect nucleotide transport (nigericin catalyses K^{+}/H^{+} exchange across membranes). More recently Weber and Winkler (1981) showed that the energy dependent and non energy dependent uptake processes could be separated by use of permeant anions to collapse the membrane potential, although whether the 'diffusion' that these authors noted in the presence of thiocyanide was atractyloside sensitive was not investigated. Provisionally, these results have been confirmed using chromaffin granule ghosts (Gruninger et al., 1981). The specificity of the carrier was also investigated by Weber and Winkler, who showed that the carrier had a broad specificity for nucleotides, as had been previously demonstrated by Phillips and Morton (1978). This suggests that the granule nucleotide content simply reflects the cytoplasmic concentration of the various

nucleotides. In this respect, as well as the absence of an obligatory nucleotide exchange, the granule transport system differs from the adenine nucleotide translocator of mitochondria.

1.5.3. Ascorbate transport

Ascorbate was first shown to be present in the chromaffin granule matrix in 1975 (Terland and Flatmark), at a concentration of 13 mM. Its presence was sought as it had previously been shown to be an electron donor to dopamine β-hydroxylase. In a later work Ingebretsen et al. (1980) suggested that ascorbate may be actively transported as the ratio of granule to cytoplasmic ascorbate was about 4:1. However Tirrell and Westhead (1979) showed, using oxidised cytochrome c as an electron acceptor and ^{14}C-ascorbate, that dehydroascorbate was most probably taken up and reduced inside the granule, by electron transport from an unknown reductant. The uptake process was shown to be independent of the proton gradient and was only partially inhibited by reducing the temperature to 0^{o}C. Clearly more work is needed to elucidate the precise transport and redox role of intragranular ascorbate.

1.5.4. Cation transport

It was shown by Borowitz et al. (1965) and Phillips et al. (1977) that Ca^{2+} and Mg^{2+} are stored within the chromaffin granule in high concentrations relative to the cytoplasm. Initial work on the nature and origin of the calcium store (Serck-Hanssen et al., 1973) presented evidence that Ca^{2+} is taken up into the granule. By perfusing adrenal glands with acetylcholine and Ca^{2+}, they were able to allow Ca^{2+} ions to enter the cell as well as stimulating exocytosis. They showed that recently emptied vesicles took up Ca^{2+} very quickly, achieving concentrations as high as those found in granules. They suggested that this could serve to control the intracellular Ca^{2+} levels.

Johnson and Scarpa (1976), working on isolated granules, suggested that Ca^{2+} was not taken up although their methods lacked sensitivity and their external Ca^{2+} concentrations were low (500 μM). Kostron et al. (1977) showed that in intact granules $^{45}Ca^{2+}$ was taken up, although because of the high internal Ca^{2+} concentration it was not clear whether $^{45}Ca^{2+}$ was simply exchanging with internal Ca^{2+}. They also showed that Ca^{2+} uptake was not ATP stimulated, which argued against divalent cations being transported as a complex with ATP. The problems with exchange were resolved, recently, by the use of chromaffin granule ghosts (Phillips, 1981). Although uptake of $^{45}Ca^{2+}$ was not much greater than residual bound Ca^{2+} most of it could be released by osmotic shock suggesting genuine uptake, rather than a binding phenomenon. ATP inhibited uptake, probably by chelation of Ca^{2+}, whilst MgATP may have inhibited uptake by creation of a membrane potential (inside positive). Phillips also demonstrated that a Ca^{2+}/ Na^{+} exchange process could account for the observed uptake.

1.6.1. The chromaffin granule Mg^{2+} dependent ATPase

Work concerned with catecholamine transport, described earlier, showed that catecholamine uptake was driven by a proton gradient, which was maintained by a proton translocating ATPase. Work on the ATPase has fallen into two areas: mechanistic and structural. The presence of an ATPase in the chromaffin granule was discovered by Hillarp (1958) based upon an earlier observation (Hillarp and Falck, 1956) that intragranular ATP was hydrolysed only when chromaffin granules were lysed. This indicated that the ATPase activity was located on the cytoplasmic face of the chromaffin granule membrane. Although other authors (Fortier et al., 1959) attributed the activity to contamination, Banks (1965) using sucrose density gradient centrifugation to obtain pure granule membranes, and Kirshner et al. (1966a) using the sulphydryl

modification reagent N-ethylmaleimide showed that the ATPase activity was distinct from that of other fractions. Banks also showed a requirement for a divalent cation to obtain ATPase activity. More recently Laduron et al. (1976) suggested that the granule ATPase may be artefactual as ATPase activity was found in a fraction other than chromaffin granules using continuous sucrose density gradient centrifugation. However Njus and Radda (1978) suggested the effect observed by Laduron et al. was due to hypotonic media, which caused the granules to lyse.

The function of the ATPase has been a subject of much dispute. Early work suggested that the ATPase may be associated with exocytosis, as addition of Mg ATP to chromaffin granules caused lysis of the granule. However an alternative explanation was that the ATPase was involved in catecholamine uptake as Mg ATP was shown to have a marked stimulatory effect on catecholamine uptake into granules (Kirshner, 1962). It was not until the use of chromaffin granule ghosts that the true nature of the ATPase was discovered. Phillips (1974) working with ghosts demonstrated that catecholamine uptake was stimulated by ATP hydrolysis and proposed a catecholamine transporting ATPase but found it difficult to explain the ratio of ATP hydrolysed to catecholamines transported of 1 to 250. This problem was resolved by Raddas group in Oxford (see earlier) who proposed a proton translocating ATPase for the granule.

Several methods have since been used to demonstrate proton translocation across the chromaffin granule membrane. Using the fluorescent probe ANS whose fluorescence is altered by binding to biological membranes and whose binding can be correlated with transmembrane electrochemical gradients, the Oxford group (Casey et al., 1976; Bashford et al., 1975, 1976b) demonstrated several changes in ANS fluorescence predicted by electrogenic proton translocation. Firstly

the fluorescence quantum yield is a function of pH and therefore addition of the uncoupler N-3-t-butyl-5 chlorosalicyl-2-chloro-4-nitroanilide(S-13) which is known to translocate protons across membranes should alter the pH gradient until the external and internal pHs are equal. This alters the ANS fluorescence, unless the pH outside the granule is the same as that inside. By this method the internal pH of the chromaffin granule was shown to be 5.5. Secondly in the absence of uncouplers the fluorescence could be increased by the addition of Mg ATP to the external medium in the presence of a permeant anion. This effect was abolished by S-13. Thirdly, ATP-dependent lysis of granules was shown to be osmotic in origin, as the net effect of ATP and $MgCl_2$ was to pump HCl into the granule, increasing the internal osmolarity.

The proton pumping effect was also measured by use of ^{31}P-nuclear magnetic resonance spectroscopy (NMR) by Radda's group (Casey et al., 1977; Njus et al., 1978). The γ-phosphate of ATP has a pK at about the pH of the granule matrix, at which point the degree of protonation of the γ-phosphate shifts dramatically, this markedly affecting the NMR properties of the γ-phosphate (principally a shift in the peak frequency). By reconstituting the internal matrix, or concentration of the material released by osmotic shock, and titrating with acid and base the $\gamma^{31}P$ peak versus pH and the buffering capacity of the matrix could be calculated. Also ATP hydrolysis could be followed by appearance of inorganic phosphate. This technique showed that ATP hydrolysis was coupled to proton translocation. By allowing for proton leakage Njus et al. (1978) demonstrated that the ratio of protons pumped to ATP hydrolysed was 2. However large pH changes could not be measured in chromaffin granules as the internal buffering capacity is too high to see any noticeable effects in the absence of

permeant anions (as the potential built up inhibits ATPase activity) whilst use of permeant anions led to lysis of the granules.

Using ghosts Flatmark and Ingebretsen (1977) were able to follow pH changes upon MgATP addition, ATPase being assayed by high performance liquid chromatography and pH being monitored by bromothymol blue sealed inside the ghosts. They showed that ATP hydrolysis was coupled to a decrease in the internal pH of up to 1.7 pH units and obtained an H^+/ATP ratio of 1.58. However these authors did not consider the effect of proton leakage in their calculations. Phillips and Allison (1978) showed the requirement of permeant anions for transport of protons in ghosts and measured a pH change of 1.53 units using methylamine distribution.

More recently the requirement for permeant anions has been the basis of a suggestion that anion transport is directly coupled to proton fluxes (Creutz et al., 1980) and inhibitors of transport of anions in erythrocytes (the disulphonic stilbenes DIDS and SITS) have been shown to inhibit ATPase activity. The effect was not abolished by the detergent Lubrol WX although FCCP-dependent activation of ATPase activity was (Creutz et al., 1981), which suggests that Lubrol uncoupled the membrane. Phillips and Allison (1978) suggested that the ATPase might function as a OH^-/anion exchange protein. The effects observed by Pollard's group are unclear and will require further investigation, as the detergents which allowed proton fluxes could well allow anion fluxes and may also uncouple any ATPase activity. At present the evidence presented here still favours an electrogenic proton-translocating ATPase as the theory of Pollard and coworkers fails to explain the observation of Johnson and Scarpa, that in the absence of a permeant anion a membrane potential could be created by MgATP.

The ATPase activity has been reconstituted into proteoliposomes

Inhibitor	Mitochondrial ATPase	Chromaffin granule ATPase	Reference
Oligomycin	+	-	1, 2
Aurovertin	+	-	1, 3
Efrapeptin	+	-	1, 2
Quercetin	+	+	1, 2
DCCD	+	+	1, 4
Alkyl tins	+	+	1, 5
Silicotungstate	+	+	5, 6
N-ethyl maleimide	-	+	7, 8

Table 1.2. Comparison of the inhibitor sensitivity of chromaffin granule and mitochondrial ATPases

+ indicates sensitivity to inhibitor, whilst - does not.

(1) Linnett and Beechey, 1979; (2) Apps and Glover, 1978; (3) Apps and Schatz, 1979; (4) Bashford et al. 1976; (5) Apps et al. 1980; (6) Racker, 1976; (7) Senior, 1979; (8) Kirshner et al., 1966.

by two groups (Buckland et al., 1979, 1981; Roison et al., 1980) after solubilization with cholate and ATP dependent proton translocation demonstrated in the reconstituted vesicles. Nonionic detergents have been used to solubilise the ATPase complex and estimate its molecular weight. Values of 150,000 (Ingebretsen and Flatmark, 1977) and 400,000 (Apps and Reid, 1977) have been obtained. Finally ATP synthesis has been demonstrated (Taugner et al., 1980; Scherman and Henry, 1980) by imposing a $\Delta\psi$ and pH gradient across ghosts in the presence of ADP and phosphate.

1.6.2. Inhibitor sensitivity and structure of the Mg^{2+} ATPase

The inhibitor sensitivity of the ATPase can be used to make comparisons with other ATPases; this can be seen in Table 1.2, which compares the mitochondrial ATPase with chromaffin granule ATPase, The data is derived where indicated from Linnett and Beechey (1977) and Racker (1976) for mitochondrial inhibitor sensitivity, Kirshner (1962) Apps and Glover (1978), Apps and Schatz (1979) and Apps et al. (1980b) for chromaffin granule inhibitor sensitivity. As can be seen oligomycin, efrapeptin and aurovertin can be used to distinguish between the two ATPase activities as they fail to inhibit the chromaffin granule ATPase. N-ethylmaleimide inhibits the chromaffin granule ATPase but not the mitochondrial ATPase. Other inhibitors affect both ATPases although Nbf-Cl may be acting to inhibit chromaffin granule ATPase (Price and Radda, 1972) at a sulphydryl residue as the granule ATPase is N-ethylmaleimide sensitive whereas the mitochondrial F_1 ATPase is not inhibited by N-ethylmaleimide (Senior, 1979), Nbf-Cl inhibiting at a tyrosine residue (Ferguson et al., 1975). Although aurovertin inhibits soluble fractions of the ATPases from adrenal mitochondria and chromaffin granules, only the mitochondrial ATPase enhanced aurovertin fluorescence (Apps and Schatz, 1979). Of major importance for work in this thesis was the observation of Bashford et al. (1976) that DCCD

inhibits the membrane bound chromaffin granule ATPase. The inhibition of both mitochondrial and chromaffin granule ATPases by DCCD and trialkyl tins (Apps et al., 1980b) suggests a similarity in structure of both ATPases. Both these inhibitors have been shown to inhibit mitochondrial ATPases by binding to the membrane sector of the ATPase; DCCD binding covalently to an organic solvent soluble protein (see later).

Water soluble ATPase activity can be removed from mitochondrial particles by chloroform treatment of the membranes (Beechey et al., 1975; Stutterheim, et al.1980) and chloroform has also been shown to extract ATPase activity from chromaffin granules (Apps and Glover, 1978). These authors obtained a higher specific activity of their ATPase using dichloromethane. Apps and Schatz (1979) were able to purify the chromaffin granule ATPase and make comparisons between it and the mitochondrial F_1 ATPase. Proteolytic fingerprints of the β and γ chains (see later) showed homogeneity with mitochondrial β and γ chains, but the α chain showed some minor peptides not present in the mitochondrial α chain. Antibodies raised against yeast and beef heart F_1 also showed cross reactivity with chromaffin granule F_1 although quantitative microcomplement fixation showed different titres for the three ATPases.

1.7.1. Proton translocating ATPases

From the preceding pages it is clear that the chromaffin granule membrane ATPase is very similar in structure and function to mitochondrial ATPase. In this section a brief review of the structure and function of the mitochondrial ATPase is therefore necessary. The ATPase can be divided into two distinct sectors, a catalytic sector known as F_1 (coupling factor $_1$, Penefsky et al. 1960), and a membrane bound segment known as F_o (because of its sensitivity to oligomycin). These two segments could be distinguished by their inhibitor sensitivity, some being assigned to F_1 ATPase such as aurovertin, Nbf-Cl and silicotungstate

and some to F_o such as oligomycin, DCCD and trialkyl tins (Linnett and Beechey, 1979).

1.7.2. F_1 ATPase

A soluble component, which catalyses the hydrolysis of ATP but is unable to catalyse ATP synthesis or ATP-phosphate exchange, unless reconstituted with its membrane, has been isolated from several energy transducing membranes such as those of mitochondria, chloroplasts and bacterial cell walls. This factor can be isolated from mitochondria by several treatments such as sonication (Penefsky, 1979) or organic solvent treatment (Beechey et al., 1975; Fisher et al., 1981) and has been purified to homogeneity. Several reviews on the subunit structure and structure-function relationships of the F_1 have been published (Senior, 1973, 1979; Futai and Kanazawa, 1980; Nelson, 1981) and this section will summarise their work. F_1 is composed of five subunits, in order of decreasing size, α β γ δ ε. They are organised as a soluble globular protein, molecular weight 325,000 in chloroplasts (Farron, 1970) to 380,000 in liver mitochondria (Catterall and Pedersen, 1971) although variation in the molecular weight estimates is probably due to the method of isolation of the F_1 fraction as well as the system of study. Based upon coomassie blue staining, Brooks and Senior (1971) obtained a subunit stoichiometry of α_3 β_3 γ δ ε, which was confirmed by Catterall et al. (1973) and shown by Yoshida et al. (1975) to be similar to that of F_1 from the thermophilic bacterium PSIII. Based upon cysteine stoichiometry Senior (1975) suggested an α_2 β_2 γ_2 δ_2 ε_2 stoichiometry. More recently Bragg and Hou (1979) proposed a stoichiometry of α_3 β_3 $\gamma\delta\varepsilon$ based upon labelled amino acid incorporation into bacterial F_1. It seems likely that due to conservation of structure all the F_1 ATPases have a similar stoichiometry and so evidence tends to favour α_3 β_3 γ δ ε, although Nelson (1981) presents

evidence for an $\alpha_2\ \beta_2\ \gamma\ \delta\ \varepsilon_{1-2}$ stoichiometry in chloroplast F_1. For the molecular weight of the individual subunits, the values quoted by Futai and Kanazawa (1980) are used α = 62 000-57 000, β = 56 000 - 50 0000, γ = 37 000-31 000, δ = 18 000-15 000 and ε = 13 000-5 700. The δ and ε subunits have been shown to be required for F_1 F_o binding (Futai and Kanazawa, 1980) whilst α β and γ have been shown to have ATPase activity when reconstituted from individual subunits.

1.7.3. The F_o segment of H^+-translocating ATPases

The F_o has been shown to comprise three to five subunits which have been termed $\chi\ \psi\ \Omega$ or a, b, c (Fillingame 1981) in bacteria I, II III in chloroplasts (Nelson, 1981) and factor B in mitochondria (Senior, 1979). Of these membrane bound subunits only one, the DCCD reactive protein, has been well characterised. Mitochondria depleted of F_1 have been shown to be 'leaky' to protons and this leakiness could be reduced by addition of F_1 to submitochondrial particles (Racker, 1976). Thus the role of F_o was thought to provide a proton channel for the ATPase. Recently Negrin et al. (1980) and Friedl and Schairer (1981) have separated F_1 from F_o using purified F_oF_1 complex from Escherischia coli and after reconstitution of F_o into liposomes demonstrated proton conduction. The proton conductivity of these membranes could be removed by the addition of DCCD or F_1 to the proteoliposomes. This follows work on PSIII F_o (Okamoto et al. 1977) where proton conduction had been shown in proteoliposomes containing purified F_o. Nelson (1981) suggests that subunit III comprises the proton channel whilst subunits I and II function to stabilise the channel. This view of the proton channel is confirmed by Fillingame, 1981. It appears that subunit b, II(ψ) may function to join F_1 to F_o. Subunit III has been shown to be the site of reactivity of DCCD.

1.7.4. The DCCD reactive proton

In a recent review Sebald and Hoppe (1981) summarise most of the work on purification, and characterisation of the DCCD reactive protein whilst Fillingame (1981) and Nelson (1981) summarise the functional aspects of this protein.

Beechey et al. (1966) first introduced DCCD as an inhibitor of oxidative phosphorylation and later showed that DCCD bound covalently to a low molecular weight subunit of the ATPase which was soluble in organic solvents (Cattell et al. 1971) (see also chapter 5). Although the effects of carbodiimides are essentially nonspecific the only proteolipid reacting in many energy transducing membranes was shown to be part of the ATPase complex (Altendorf, 1977; Sigrist et al. 1977; Sone et al. 1979; Sebald et al. 1979b). This protein has been sequenced from a number of different sources such as E. coli, PSIII and Mastigocladus laminosus plasma membranes, Neurospora crassa, Saccharomyces cerevisiae and beef heart mitochondria, and chloroplasts (Sebald and Hoppe, 1981). These authors have also predicted the secondary structure from the sequence analysis and shown four α helices broken by two β turns, and have derived an evolutionary tree similar to those derived for cytochrome c. The site of DCCD modification has always been shown to be a single carboxyl amino acid at position 65 which is one of six conserved amino acids, and mutants of E. coli lacking DCCD sensitivity have been shown to have a glycyl residue in position 65 (Wachter et al., 1980).

The DCCD reactive protein from PSIII (Sone et al. 1979), chloroplasts (Nelson et al. 1977; Sigrist-Nelson and Azzi, 1980) and beef heart mitochondria (Celis, 1980) has been reconstituted into liposomes, whilst the yeast mitochondrial DCCD reactive protein has been reconstituted into planar lipids formed across a millipore filter

(Criddle et al. 1977). Nelson et al. (1977) incorporated the butanol extract from chloroplasts into liposomes with bacteriorhodopsin. Light dependent proton pumping was measured in the presence and absence of DCCD. DCCD caused an increase in H^+ uptake which was interpreted as a decrease in proton leakage through the proteolipid. Sigrist-Nelson and Azzi (1980) have since used both quenching of 9-aminoacridine fluorescence and pH electrode measurement to follow H^+ conduction in proteoliposomes reconstituted with DCCD-reactive proteins and have shown that the liposome is 'leaky' to protons, and the proton conductance is DCCD sensitive. Celis (1980) used aminoacridine fluorescence to follow H^+ conduction and was able to show DCCD sensitivity. Sone et al. (1979) showed similar results using the PSIII plasma membrane proteolipid, which was purified by chloroform:methanol extraction. Criddle demonstrated an oligomycin sensitive increase in conductance in bilayer lipid membranes upon incorporation of chloroform:methanol extract of yeast mitochondria into bilayers. Fillingame (1980,1981) however, questions the role of the DCCD reactive protein in forming the proton channel as the chloroform: methanol extract from E.coli plasma membrane does not show proton conducting properties. This may be due to the purification procedure used or to the reconstitution conditions, as chloroplast proteolipid has been shown to require galactolipids for maximal activity.

The stoichiometry of the DCCD reactive protein in relation to the ATPase has been a subject of considerable dispute. Estimates have been based upon the DCCD sensitivity of the ATPase, which showed that sub stoichiometric amounts of DCCD to proteolipid were required to maximally inhibit the ATPase (Fillingame 1976; Sigrist-Nelson et al. 1978; Sebald et al. 1979a; Sone et al. 1979). In fact estimates of DCCD:DCCD-reactive proteins give one DCCD bound to every three DCCD-reactive proteins from the ATPase (Fillingame et al. 1976) or every six DCCD-reactive subunits

(Sebald et al. 1979a). This, coupled with work on the chloroplast proteolipid using a spin labelled analogue of DCCD, (NCCD), showed spin-spin interaction between NCCD molecules when one NCCD was bound to every three proteolipids (required for maximal inhibition of ATPase). The spin-spin interaction was suggested to be due to two NCCD binding to one ATPase and a stoichiometry of 6 proteolipids per ATPase derived. This is supported by work measuring the incorporation of ^{3}H amino acids (Sebald et al., 1979a), which showed a six to seven fold stoichiometry for the DCCD reactive protein.

1.8.1. Other proton translocating ATPases

Since it is by no means certain that the chromaffin granule ATPase is of the F_oF_1 type this section contains a brief review of other H^+-translocating ATPases. The most thoroughly investigated ATPase of this type has been isolated from fungal plasma membranes. This follows from the observation of Slayman (1965) that, in N. crassa, during fermentative glycolysis, the pH of the medium drops by 2-3 units. The kinetics and inhibitor sensitivity of this ATPase activity have been recently reviewed by Goffeau and Slayman (1981) and so this review will briefly summarise some of the results. The ATPase has been isolated from the plasma membranes of Schizosaccharomyces pombe, N. crassa, S. cerevisiae and Candida tropicalis. The kinetic work shows the presence of a phospho-protein intermediate during the proton translocating cycle. The ATPase is not inhibited by oligomycin, venturicidin or trialkyltins but is highly sensitive to vanadate, DIDS, mercurials, quercetin, leucinostatin and triaryl sulphonium ions. It is partially sensitive to DCCD (I_{50} = 50 μM). The pure protein consists of a single polypeptide chain, M_r = 105 000, but the presence of a small proteolipid subunit has not been excluded by previous work.

The vacu olar membrane of S. cerevisiae has also recently been purified (Ohsumi and Anraku, 1981), and has been shown to contain a proton translocating ATPase. The ATPase can be differentiated from both the mitochondrial and plasma membranes, by its pH optimum insensitivity to azide or vanadate, and its differential sensitivity to oligomycin and DCCD (Kakimura et al. 1981). At present there has been no molecular characterisation of this ATPase.

Finally an ATPase has been isolated from the gastric mucosa of hogs, rats, rabbits and humans. This has been shown to catalyse electroneutral K^+/H^+ exchange (Wallmark et al. 1980) forming a phosphoprotein intermediate. It is inhibited by DCCD (Chang et al. 1978) and irradiation (Saccomani et al. 1981). On SDS polyacrylamide gels it appears to have a molecular weight of 90-105000, whilst target size irradiation indicates an active complex of M_r = 270000. Thus the enzyme probably functions as a trimer (Saccomani et al. 1981).

1.8.2. Other protein complexes modified by DCCD

Apart from ATPases, DCCD has also been shown to inhibit other complexes of the mitochondrial membrane. Cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, E.C.1.9.3.1), a seven subunit complex in mitochondria is inhibited by DCCD, probably by covalent modification of subunit III (Casey et al., 1980b). Both subunits III and IV were covalently modified, but Prochaska et al. (1981) have shown that subunit II is also susceptible to DCCD binding. Low levels of DCCD blocks proton translocation by this enzyme but not electron transport (Casey et al. 1980b). The enzyme has been shown to translocate protons by Krab and Wikstrom (1978), and the binding of DCCD to subunit III has led to a hypothesis that subunit III forms the proton channel. However Ludwig and Schatz (1980) have isolated a two

subunit cytochrome oxidase from Paracoccus denitrificans which translocates protons when reconstituted into proteoliposomes (Solioz et al. 1982). The two subunits resemble subunits I and II of the mitochondrial protein in molecular weight and polarity. The DCCD reactive cyanogen-bromide peptide from subunit III has been isolated from the mitochondrial cytochrome c oxidase and shows remarkable sequence homology when compared to the amino acid residues surrounding the DCCD-reactive site of the E. coli DCCD-reactive protein (Sebald et al. 1979b; Prochaska et al. 1981).

The transhydrogenase from mitochondria which catalyses the transfer of hydrogen from $NADPH_2$ to NAD^+ and also translocates protons across the membrane is also inhibited by DCCD (Pennington and Fisher, 1981). This enzyme probably functions as a dimer within the membrane. Proton translocation could be inhibited by binding of a single DCCD molecule whilst the transfer of protons from NADPH to NAD required two DCCD molecules to be bound to each complex, one on each dimer. These results contrast with the work of Phelps and Hatefi (1981) who showed that a DCCD:subunit stoichiometry of 1:1 was needed to inhibit proton translocation and hydrogen transfer.

DCCD has been shown to inhibit the F_1 ATPase from E. coli and beef heart mitochondria (Pougeois et al. 1979) by binding to an acidic amino acid residue on the β-chain. More recently the DCCD modified cyanogen bromide peptide has been purified and sequenced from the E. coli β-chain (Esch et al. 1981). Yoshida et al. (1981) has reported a tryptic peptide of similar sequence using the DCCD-modified F_1 ATPase from PSIII plasma membranes.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Chemicals

General laboratory chemicals and solvents were analar grade, and were purchased from British Drug Houses Ltd., Poole, Dorset (BDH), with the exception of those listed below.

Chemicals. Adenosine diphosphate, disodium salt (ADP); adenosine-5'-O-(3-thiotriphosphate), trilithium salt (ATPγs); adenylyl-imidodiphosphate, tetralithium salt (ADPNP); cytidine-5'-triphosphoric acid (CTP); dithiothreitol; guanosine-5'-triphosphate, disodium salt (GTP); glyceraldehyde-3-phosphate; nicotinamide adenine dinucleotide-reduced (grade I); phosphoenolpyruvate sodium salt; 3-phosphoglyceric acid; uridine-5'-triphosphate, trisodium salt (UTP) were purchased from Boehringher Mannheim, Bell Lane, Lewes, Sussex.

Adenosine triphosphate, disodium salt (grade I) and 4-(2-hydroxyethyl)-piperazine-ethane-sulphonate (Hepes) were purchased from Sigma Chemicals, Poole, Dorset.

Solvents. Chloroform (analar) and isopropanol were purchased from Fisons.

2.1.2. Chromatography

Sephacryl 300 and Sephadex LH20 were purchased from Pharmacia (Uppsala, Sweden). Polyamide plates were purchased from BDH. DE81 paper was purchased from Whatman and cellulose nitrate filters (0.45 μM pore size) from Sartorius (Gottingen, Germany).

2.1.3. Detergents

Cholic acid, sodium deoxycholate, Nonidet P40, and Triton X-100 were purchased from BDH. Cholic acid was recrystallised from ethanol: water, 70:30. Triton X-100 was scintillation grade.

Brij 36T was bought from Canamex Chemicals (Mexico). Nonidet P42 was purchased from Shell Chemicals. Octaethylene glycol dodecylether ($C_{12}E_8$) was obtained from the Kouyoh Trading Co., Tokyo.

Digitonin, Lubrols PX and WX, egg yolk LPC, β-octyl glucoside (1-O-n-octyl-β-D-glucopyranoside), Saponin, sodium taurodeoxycholate and Tweens 20, 80 and 85 were purchased from Sigma.

2.1.4. Gel electrophoresis

Acrylamide (technical grade) and N,N'-methylene bisacrylamide were purchased from BDH. N,N'-methylene bisacrylamide was recrystallised from boiling acetone and acrylamide from ethyl acetate. Salicylic acid (reagent grade) and urea (Aristar grade) were purchased from BDH.

pH range 3.5-10 and 9-11 ampholines were purchased from LKB (LKB Produkter AB, Bromma, Sweden) and pH 4-9 from Bio Rad (Lewes, Sussex). The polyacrylamide used was initially purchased from BDH, but after BDH changed its source of supply Aldrich polyacrylamide (5,000,000-6,000,000 molecular weight) was used.

Gels were initially stained with Coomassie brilliant blue R250, but after its withdrawal from BDH catalogues, Kenacid (BDH) was used. Sudan Red IV was obtained from Gurrs (BDH).

2.1.5. Scintillants

All reagents were scintillation grade and obtained from BDH or Kochlight. Scintillation fluid contained 5 g l^{-1} of 2,5-diphenyloxazole (PPO) and 0.3 g l^{-1} of 1,4-bis-(-5-phenyloxazol-yl)-benzene (POPOP) dissolved in toluene. Triton based scintillation fluid was made by mixing two parts of toluene scintillant with one part of Triton X-100.

2.1.6. Radiochemicals

^{32}Phosphate and ^{14}C-tyramine were obtained from the Radiochemical Centre, Amersham, Bucks. ^{14}C-DCCD (50 Ci/mol) was purchased from

Gif-Sur-Yvette, CNRS, France.

2.1.7. Enzymes

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate NAD oxidoreductase EC 1.2.1.12); 3-phosphoglycerate kinase (ATP:3 phospho D-glycerate 1-phosphotransferase EC 2.7.2.3); hog muscle lactate dehydrogenase (Lactate:NAD oxidoreductase EC 1.1.1.27); rabbit muscle pyruvate kinase (ATP:pyruvate 2-O-phospho-transferase EC 2.7.1.40) were purchased from Boehringher Mannheim.

2.1.8. Proteins

Horse spleen apoferritin was a gift from Dr. D.K. Apps. Hog pepsin and trypsin were gifts from Dr. A.P. Ryle. Chicken egg white lysozyme (twice recrystallised) was purchased from Armour Chemicals Co. Ltd., Eastbourne, Sussex. Porcine insulin and glucagon were obtained from Eli Lily and Co. Ltd., Basingstoke, Hants. Bovine serum albumin (fatty acid free crystals and fraction V), bovine pancreatic α-chymotrypsin (type II), ribonuclease (type IIIb), rabbit Ɣ-globulin, and sperm whale myoglobin were acquired from Sigma.

2.2. Methods

2.2.1. Preparation of subcellular fractions

(a) Chromaffin granule membranes. The whole procedure was carried out at 0°C. Bovine adrenal glands were obtained soon after slaughter and were immediately placed on ice. Approximately 30-40 glands were sliced in half and the medullae scraped out using a sharp scalpel. The medullae were placed in 250 mls of 0.3 M sucrose buffered with 10 mM Hepes-NaOH pH 7.4 (all solutions were buffered with 10 mM Hepes-NaOH pH 7.4 (Hepes buffer) unless otherwise stated). The medullae were then minced and passed three times through a motor driven Potter-Elvehejm homogeniser

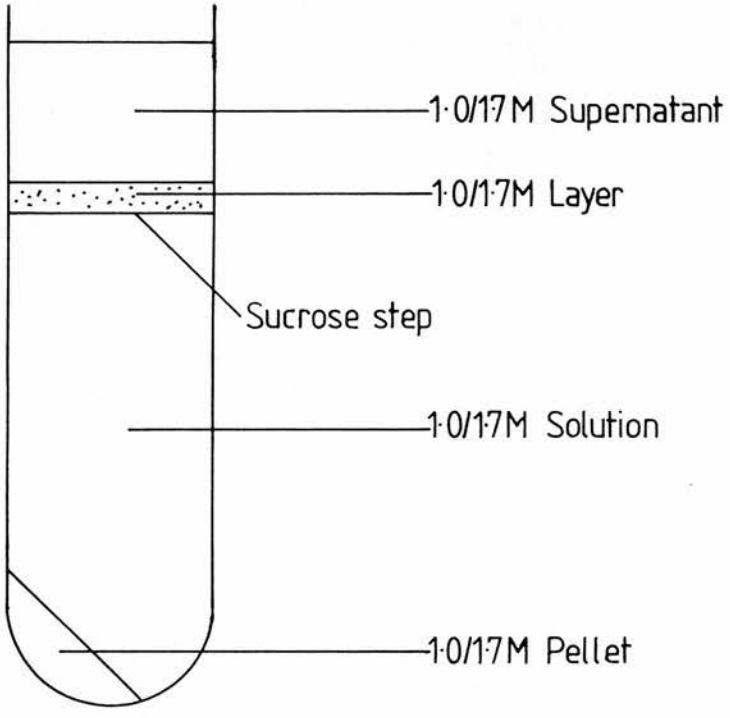


Figure 2.1. Sucrose step density gradient centrifugation

The diagram demonstrates the fractions that were collected after centrifugation of the step gradients of sucrose as described in Chapter 2.

rotating at approximately 500 r.p.m. The homogenate was diluted to 1 l with 0.3 M sucrose/Hepes buffer and centrifuged at 4000 r.p.m. for 5 minutes (Beckman JA14 rotor). The cell debris pelleted during this centrifugation was discarded. The supernatant (termed 4K supernatant subsequently) was centrifuged at 14 000 r.p.m. for 30 minutes (Beckman JA14 rotor) to pellet mitochondria and chromaffin granules. The supernatant (termed 14K supernatant subsequently) was discarded and the pellet (termed 14K pellet) resuspended in 0.3 M sucrose/Hepes buffer using the homogeniser at about 200 r.p.m. The volume was made up to 120 mls and 20 ml portions layered onto 50 mls of 1.7 M sucrose/Hepes buffer in Beckman 70 ml centrifuge tubes, care being taken not to mix the layers. The tubes were centrifuged at 45 000 r.p.m. for 60 minutes (Beckman Ti 45 rotor).

The contents of the tube could be divided into four separate fractions. A diagram of a rotor tube containing step density gradient is shown in Figure 2.1, which also describes the terminology used to identify each fraction. The upper layers of the tubes were removed using a Pasteur pipette connected to a water pump, leaving the 1.7 M pellet. The sides of the tube were wiped with tissue and the pellet suspended in 50 mls of Hepes buffer, gently homogenised, diluted to 200 mls with Hepes buffer and centrifuged at 45 000 r.p.m. for 20 minutes (Beckman Ti 45 rotor). The supernatant from this step (termed the chromaffin granule lysate subsequently) was discarded and the pellet (termed the chromaffin granule lysis pellet subsequently) resuspended by gentle homogenisation in 40 mls of Hepes buffer. The resuspended pellet was divided into two, loaded onto 50 ml portions of 1.0 M sucrose/Hepes buffer in two 70 ml Beckman tubes and centrifuged at 45 000 r.p.m. for 30 minutes (Beckman Ti 45 rotor). The fractions from this step gradient could be described in an analogous way to the

1.7 M sucrose step gradient. These fractions are also shown in Figure 2.1. The 1.0 M layer contained the purified chromaffin granule membranes.

Chromaffin granule membranes could be stored at -20°C for routine work. However when it was important to minimize the rate of loss of ATPase activity, the membranes were stored in 10 mM Hepes-NaOH pH 7.4, 2 mM DTT, and 0.1 mM EDTA (Hepes/DTT/EDTA buffer) at -20°C until needed.

(b) Adrenal mitochondria. The preparation of adrenal mitochondrial membranes was an adaptation of the chromaffin granule preparation. The procedure was identical as far as the 1.7 M step gradient. The 1.7 M layer, rich in mitochondria, was suspended in 200 mls of Hepes buffer and centrifuged at 45 000 r.p.m. for 20 minutes (Beckman Ti 45 rotor). The supernatant from this centrifugation (mitochondrial lysis supernatant) was discarded and the pellet (termed mitochondrial lysis pellet subsequently) resuspended in 40 mls of Hepes buffer by gentle homogenisation. The resuspended pellet was layered over two 50 ml portions of 1.0 M sucrose/Hepes buffer in 70 ml Beckman tubes and centrifuged at 45 000 r.p.m. for 30 minutes (Beckman Ti 45 rotor). The mitochondrial 1.0 M sucrose step gradient could be fractionated in an identical way to the chromaffin granule step gradient. The mitochondrial 1.0 M pellet contained a fraction rich in adrenal mitochondrial membranes which could be stored at -70°C.

(c) Beef heart mitochondria. The method of preparing beef heart mitochondria is based upon that of Smith (1967), method (3), except that all buffers used contained 1 mM succinate to preserve mitochondrial succinate oxidase activity.

2.2.2. Linear sucrose density gradients

Further purification of membrane fractions obtained in 2.2.1 (a)

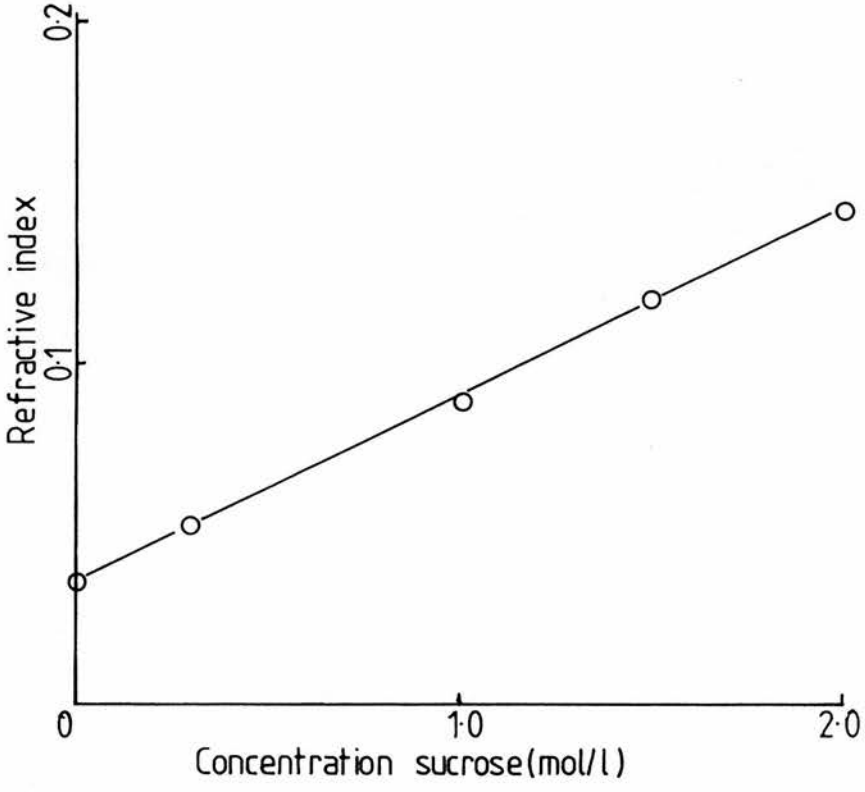


Figure 2.2. Standard curve of refractive index versus sucrose concentration

Sucrose concentration was assayed as described in the text (all media were buffered with 10 mM Hepes-NaOH pH 7.4).

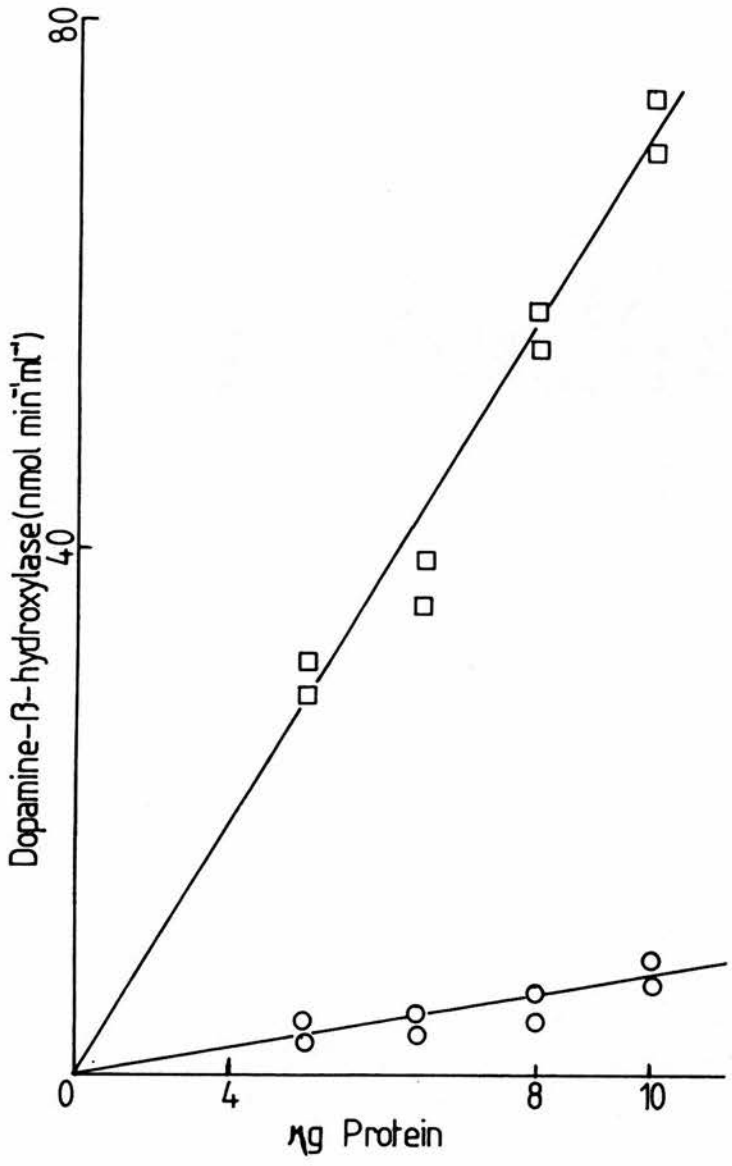


Figure 2.3. Standard curve showing dopamine β-hydroxylase activity versus protein concentration

Dopamine β-hydroxylase was assayed as described in the text. The figure shows dopamine β-hydroxylase activity versus protein concentration for chromaffin granule membranes (□) and chromaffin granule lysate (○). Protein was assayed by the method of Bradford (1976).

and (b) could be achieved by use of isopycnic sucrose density gradient centrifugation. Linear gradients of 0.5 M - 1.5 M sucrose, buffered with 10 mM Hepes-NaOH, pH 7.4, were poured at a rate of 0.5 ml/min, using a two-chamber gradient mixer and peristaltic pump. Cellulose nitrate centrifuge tubes (8.7 cm x 1.3 cm^2) were used. For purification of chromaffin granule membranes, the gradients were layered over 1.0 ml of a suspension of the membranes in 1.5 M sucrose. During centrifugation the membranes floated to their equilibrium bouyant density (about 0.8 M sucrose). This technique avoids contamination of the purified membrane band with dense, slowly sedimenting membrane fragments, which can occur with sedimentation from the top of the gradient. For purification of mitochondrial membranes, which banded at 1.3 M sucrose, loading was at the top of the gradient. The tubes were centrifuged at 41 000 r.p.m. for 180 minutes (Beckman SW41 rotor). Fractions were collected after piercing the bottom of the tubes and pumping out the contents at a rate of 1 ml/min. Sucrose concentrations were assayed using an Abbe 50 refractometer with a sodium lamp. A standard curve of refraction versus sucrose concentration is presented in Fig. 2.2.

2.2.3. Enzyme Assays

(a) Dopamine β-hydroxylase (3,4-dihydroxyphenylethylamine β-hydroxylase EC 1.14.17.1). Dopamine β-hydroxylase activity was assayed by the method of Friedman and Kaufman (1965). An exact description of the assay can be found in Abbs (Ph.D. thesis, 1980). The basic method uses ^{14}C-tyramine as substrate, and measures the product octopamine, which is chemically converted to p-hydroxybenzaldehyde by periodate. ^{14}C-p-hydroxybenzaldehyde is extracted into toluene, and measured by liquid scintillation spectrometry. A plot of enzyme concentration

against enzyme activity is presented in Figure 2.3.

(b) Cytochrome b_{561} content. The method of estimating cytochrome b_{561} content was by reduced/oxidised difference spectroscopy using a split beam spectrophotometer (Silsand and Flatmark, 1974). Two methods are presented here, one of which gives highly accurate estimates but is time consuming, whilst the other gives a less accurate but quicker method of estimating the cytochrome content.

Type I assay. The sample for assay (about 2 mg protein/ml) was dialysed twice against 200 volumes of water, adjusted to the following concentrations: protein 1 mg/ml, Tris-H_2SO_4 (pH 7.4) 20 mM, EDTA 1 mM, $C_{12}E_8$ 0.1%. 1.5 ml was divided into two equal portions in separate cuvettes and loaded into a Unicam SP 1800 spectrophotometer. 20 µl of 4 mM $K_3Fe(CN)_6$ were added to oxidise the sample in the reference cuvette, and also to another cuvette, containing 0.74 ml of buffer and placed in front of the reduced sample cuvette. A few crystals of sodium dithionite were added to the reduced sample cuvette, and the reduced versus oxidised spectrum recorded over the range 430-600 nm. The content of cytochrome b_{561} was determined from the difference between the absorbance at 561 nm and 575 nm, using an extinction coefficient 23.3 x 10^6 cm^2 mol^{-1} (Silsand and Flatmark, 1974). Dialysis of the sample improved the accuracy of the assay as absorbance differences between oxidised and reduced catecholamine produced a steep gradient in the baseline of the spectrum in the region of interest, particularly after oxidation by ferricyanide.

Type II assay. Protein solution (0.25 mg/ml) was added to two 1 ml cuvettes in an SP 1800 split beam spectrophotometer. The solution in one cuvette was reduced with a few crystals of sodium dithionite and the spectrum scanned from 450-600 nm. The absorbance difference at 561 nm was read and an extinction coefficient of 1.7 x 10^6 cm^2 mol^{-1} used to

calculate the specific content.

The type I assay, whilst being tedious to set up, gives consistently higher and probably more accurate estimates of the real cytochrome content of the sample than the more rapid type II assay, in which the assumption is made that cytochrome b_{561} is fully oxidised in untreated samples. This is probably not always the case (Apps et al. 1980c).

(c) Adenosine triphosphatase (ATP phosphohydrolase EC 3.6.1.4). Two methods of assaying ATPase activity were used. One was a direct method involving measurement of ^{32}phosphate hydrolysed from γ-^{32}P-ATP, the other a coupled assay in which ADP production was linked to NADH oxidation.

Type 1(a) Preparation of γ-^{32}P-ATP. The method used was based on that of Glynn and Chappell (1964) with the modifications introduced by England (1979) and involves the exchange of ^{32}phosphate with the γ-phosphate of ATP using glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase, followed by separation on Dowex 1 times 8 (200-400 mesh) of ^{32}phosphate from γ-^{32}P-ATP. ATP made by this method was found to be less than 1% contaminated by inorganic phosphate as judged by chromatography.

(b) Assay. The ATPase assay was performed at 37^{o}C using 2 mM ATP, 4 mM $MgCl_2$, 100 mM Hepes-NaOH pH 7.4 and enough γ-^{32}P-ATP (specific activity 1 Ci/mmol) to give 0.5 µCi in the assay mix (total volume 1.0 ml). After 15 minutes the reaction was stopped by streaking 10 µl of the assay solution onto a 3 x 22 cm strip of DEAE-cellulose paper (Whatman DE81). ^{32}Phosphate was separated from γ-^{32}P-ATP by ascending chromatography (Apps and Reid, 1977) using 0.4 M ammonium formate (pH 3.1) instead of 0.6 M. The percentage of ATP hydrolysed could be converted to enzyme activity (nmoles/min/mg protein) and Figure 2.4

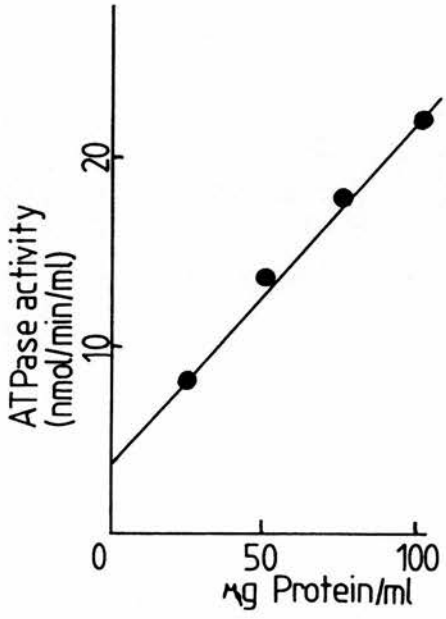


Figure 2.4. Assay of ATPase activity using release of γ-^{32}phosphate from γ-^{32}P-ATP.

The figure shows a plot of concentration of chromaffin granule membranes against ATPase activity. Protein was assayed by the method of Bradford (1976).

presents a plot of enzyme concentration against enzyme activity.

Type 2. Coupled Assay. This method in which ADP production is linked to NADH oxidation (Barnett, 1970) has a major advantage over the measurement of γ-^{32}phosphate release, in that the system regenerates ATP, thus overcoming problems which might arise from ADP acting as a competitive inhibitor of the ATPase: also it gives a continuous record of the time course of the reaction. The assay is not particularly sensitive, but it is quick and reliable, is not affected by most detergents, and has none of the problems involved in estimating inorganic phosphate. However in systems with a high residual NADH oxidase activity, such as mitochondria, the assay should not be used.

Method. The assay mixture contained 1 mM ATP, 10 mM $MgCl_2$, 50 mM KCl, 50 mM Hepes-KOH pH 7.4, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 5.5 units/ml lactate dehydrogenase, and 4 units/ml of pyruvate kinase. The substrate concentrations were saturating for the coupling enzymes; increasing them did not increase the reaction rate on addition of 10 nmoles of ADP. The cuvette holders of the spectrophotometer were thermostatted to 30°C, and cuvettes were warmed for about five minutes. The assay was then carried out and absorbance change at 340 nm read in a Gilson 400 recording spectrophotometer. The extinction coefficient for the NADH oxidation was taken as 6.22×10^6 cm^2 mol^{-1}. By use of the automatic sample change, four samples could be measured simultaneously and 30 samples per hour could be assayed. NADH oxidase activity was measured in chromaffin granule membranes in the same system, with the omission of ATP from the assay mixture, and was found to be about 10 nmol/min/mg protein. As this compared with 420-500 nmol/min/mg protein for ATPase activity, the contribution of NADH oxidase to the reaction rate could be ignored in most experiments.

(d) Cytochrome c oxidase activity. This was assayed by the method of Mason et al. (1973). 0.4 mg ml^{-1} of cytochrome c was dissolved immediately before use in 0.5% Tween 80, 40 mM potassium phosphate pH 6.65. The cytochrome was fully reduced by the addition of dithionite and excess dithionite removed by aeration. 1 ml of solution was added to each of two cuvettes. Cyanide was added to one, enzyme to both, and the rate of extinction change at 550 nm measured in a Unicam SP 1800 split beam spectrophotometer. An absorbance change of 0.1 units was taken as 5.2 nmoles of cytochrome c oxidised. Because impurities can cause oxidation of cytochrome c it was important to use very pure cytochrome, and Sigma Type IIIA was found satisfactory. Also it was important to keep the temperature constant as the assay procedure is highly temperature sensitive, and so solution to be assayed was kept at 30°C and the spectrophotometer cuvette holder was water jacketted and maintained at 30°C.

(e) Succinate dehydrogenase. An alternative mitochondrial enzyme marker was succinate dehydrogenase, and the assay procedure is based upon that of Porteous and Clark (1965) using 2 (4-iodophenyl)-3(4-nitrophenyl)-phenyl tetrazolium chloride (INT) as an electron acceptor. Enzyme to be assayed was added to 1.0 ml of 50 mM potassium phosphate, 2 mM EDTA, 1.0 mg/ml INT, 50 mM sodium succinate, pH 7.4; a control in which malonate (50 mM) replaced succinate, was included . After 30 mins at 37°C, the reaction was stopped by the addition of trichloroacetic acid (TCA), final concentration 5%, and the INT extracted into 2 ml butylacetate. Absorbance was read at 490 nm and an extinction coefficient of 20.1×10^6 cm^2 mol^{-1} used in calculations of activity.

2.2.4. Protein estimation

Two methods have been used to estimate the protein content of a

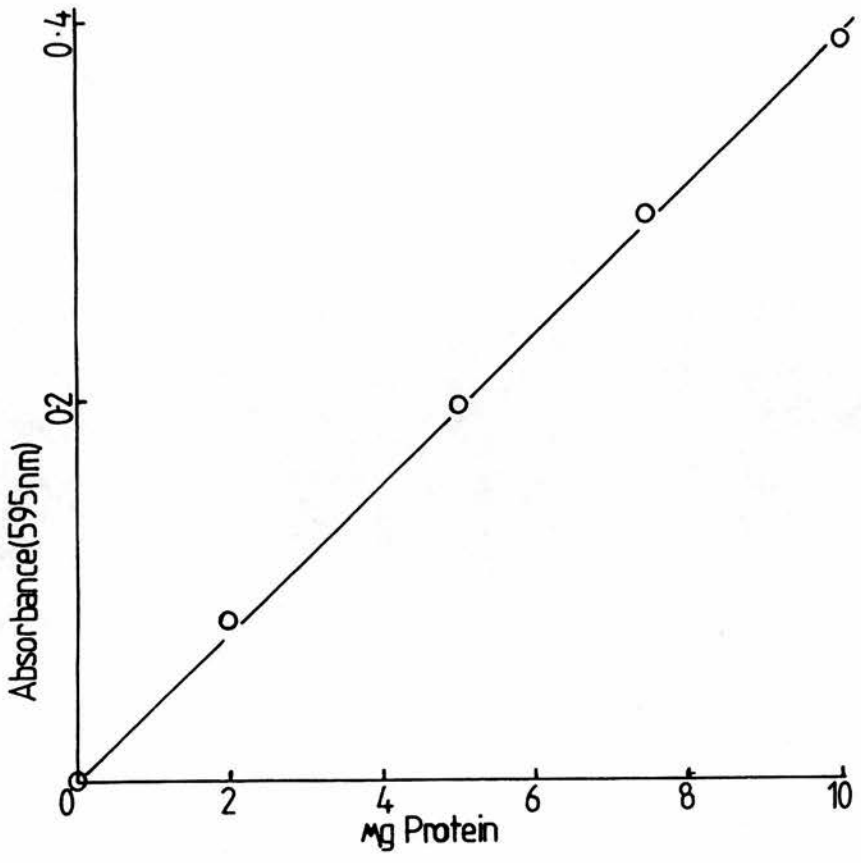


Figure 2.5. Plot of protein concentration against absorbance (595 nm) using the dye binding assay.

Protein was assayed by the modified method of Bradford (1976) using bovine serum albumin as standard.

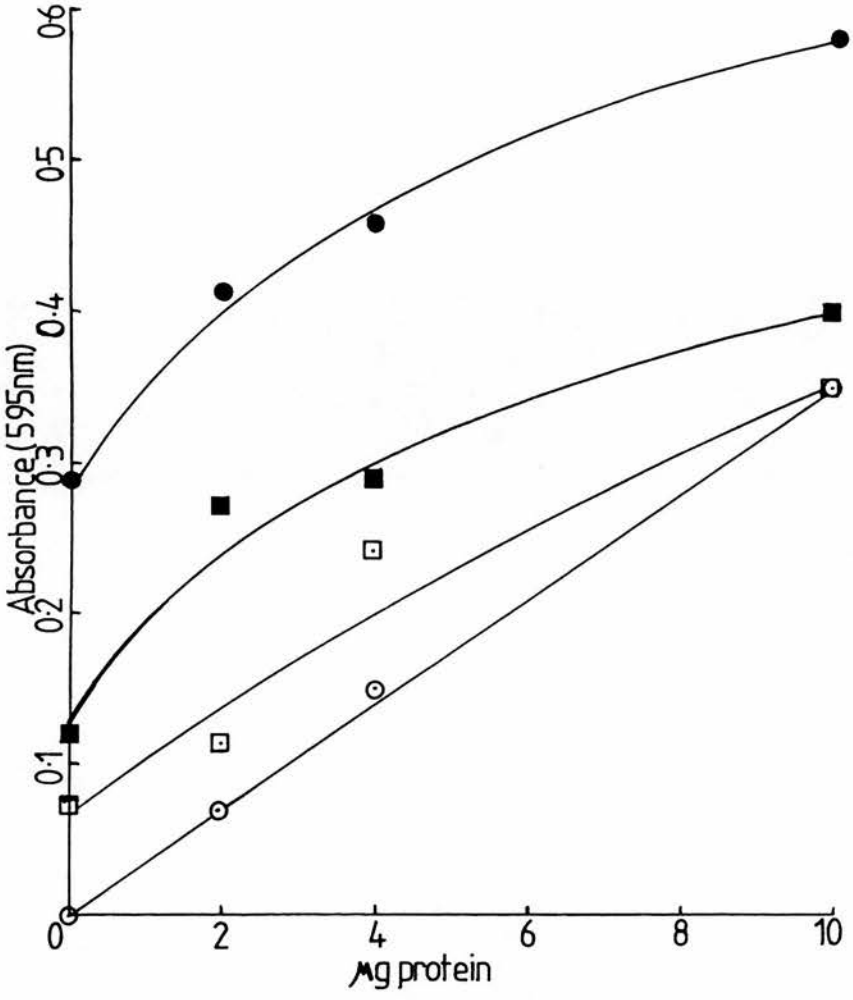


Figure 2.6 Effect of Lubrol WX on the dye binding assay.

Graph shows absorbance (595 nm) at different protein concentrations in the presence of 0 (○), 0.02 (□), 0.2 (■), and 0.5 (●) % w/w Lubrol WX using the assay of Bradford (1976).

solution, the method of Bradford (1975) and a modification of the method of Lowry (Hartree, 1972).

(a) The dye binding assay (Bradford, 1976). The actual method is exactly as described by Bradford for the microprotein assay and used bovine serum albumin as standard. However, modification of the preparation procedure increased the sensitivity some two fold compared to the original method. 10 mg of Coomassie brilliant blue G250 were dissolved in 5 ml of 95% ethanol, and left slowly stirring for 30 mins. Then 10 ml of orthophosphoric acid was added very slowly to a stirring solution and the mixture left for 3-4 hours. This was diluted to 100 ml with twice distilled water. The mixture was filtered through glass wool and cellulose nitrate paper to leave a deep brown coloured solution. This solution could be stored refridgerated for several weeks. A standard curve of absorbance at 595 nm versus protein concentration is presented in Fig. 2.5.

(b) Effect of detergent on the dye binding assay. In order to test whether the presence of detergent would affect the overall colour change the assay was carried out in the presence of 0-0.5% of the nonionic detergent Lubrol WX. The results of this experiment are presented in Fig. 2.6, and it can be seen from that figure that even very small amounts of detergent radically altered the absorbance. This assay was therefore judged unsuitable for protein estimation in the presence of detergents.

(c) Modified Lowry assay (Hartree, 1972). This was done exactly as described by Hartree (1972) except that half the volume of solution was used at each stage and the protein was dissolved in 0.1% SDS before assaying. Pretreatment of the protein by precipitation in ten volumes of either acidified acetone (1.3% with conc.HCl) or acetone:ethanol 1:1 was found to be essential, and the precipitated protein was centrifuged

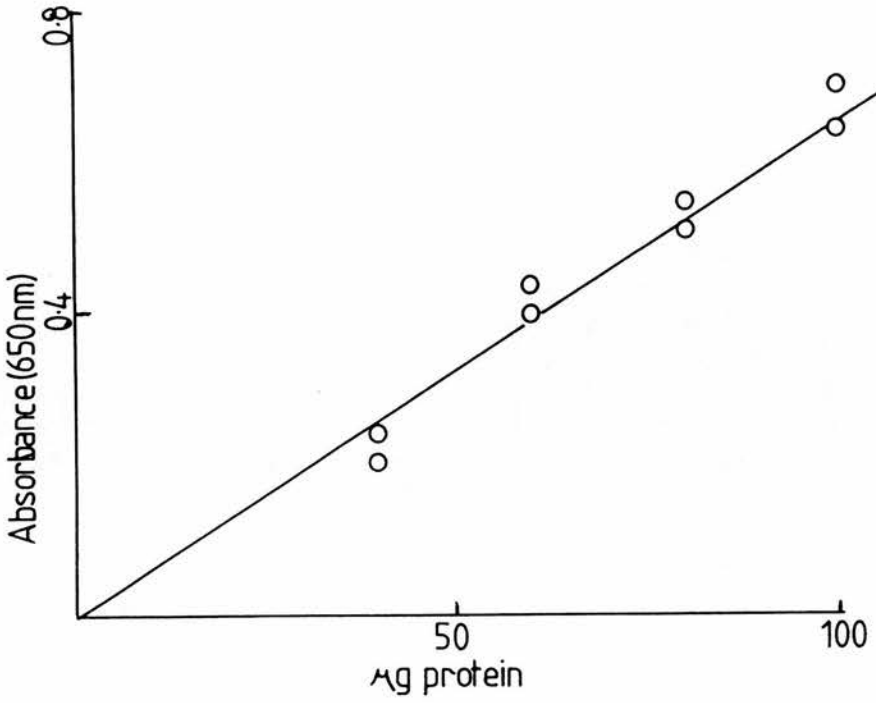


Figure 2.7 Estimation of protein concentration using the modified method of Lowry (Hartree, 1972).

Assay conditions are described in the text. Bovine serum albumin was used as standard.

Solution constituent	15% acrylamide	10% acrylamide	Stacking gel
Tris-HCl	0.375 M pH 8.8	0.375 M pH 8.8	0.125 M pH 6.8
Acrylamide (w/v)	15%	10%	5%
N,N'methylenebisacrylamide (w/v)	0.4%	0.277%	0.133%
EDTA	2 mM	2 mM	2 mM
SDS (w/v)	0.1%	0.1%	0.1%
Ammonium persulphate (w/v)	5.3×10^{-4}%	4×10^{-4}%	16×10^{-4}%
Polyacrylamide (w/v)	0.5%	0.5%	0.5%
TEMED (a) (w/v)	0.05%	0.05%	0.125%

Table 2.1. Concentration of the constituents used in SDS polyacrylamide gel electrophoresis.

The system used is described in the text.

at 10 000 r.p.m. in 1.5 ml eppendorf tubes in a Beckman Ti 45 rotor. A plot of protein concentration (bovine serum albumin) versus absorbance is presented in Fig. 2.7.

2.2.5. (a) Polyacrylamide gel electrophoresis in SDS

SDS polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (1970) using the modifications introduced by Douglas and Butow (1976) for slab gel work. The internal dimensions of the cassette were 0.12 x 14.5 x 16.2 cm. Separating gel was poured into the cassette, sealed using agar and perspex spacers, up to 3.5 cm from the top of the cassette. For gels of a constant acrylamide the composition, the separating gel was poured in using a pipette, whilst for exponential polyacrylamide gradient gels, a Gilson "Minipuls" two channel pump was used. Exponential gradients were of 10-15% acrylamide and mixed from 15 ml of 15% acrylamide in the mixing chamber, and 30 ml of 10% acrylamide in the reservoir: the pouring rate was about 1 ml/min. The exact compositions of the solutions used in SDS polyacrylamide gel electrophoresis can be seen in Table 2.1. After the separating gel was poured the top was overlaid with water saturated butan-2-ol and left to polymerise.

The butan-2-ol was thoroughly washed off with water and a stacking gel (see Table 2 for composition) poured in. To create slots for loading of proteins a spacer comb (slots contained 80-1500 µl of solution) was pushed into the polymerising stacking gel. After polymerisation the spacer comb and bottom spacer were removed and loaded into a slab gel tank (Studier, 1973) and electrophoresesed using 0.25 M Tris base, 0.1 M glycine, 0.1% SDS, 2 mM EDTA as the tank buffer. After 20 hours at 50 v (top negative) using a Shandon Vokam constant voltage-power source the bromophenol blue tracker dye had reached the bottom of the separating gel.

Samples were prepared for electrophoresis by dissolving in 0.125 M

Tris-HCl pH 6.8, 2 mM EDTA, 10% v/v glycerol, 1% w/v bromophenol blue, either two or five percent SDS, and with or without 2% v/v 2-mercapto-ethanol. The sample was then left to incubate at room temperature for 30 mins. It was not found essential to boil the samples as complete solubilisation of the protein was achieved by this procedure. Furthermore boiling of chromaffin granule membranes in SDS, with or without mercaptoethanol, leads to apparently irreversible aggregation of some proteins including cytochrome b_{561}.

A further problem encountered in the electrophoresis of chromaffin granule proteins is that low molecular weight proteins were retarded, or the bands distorted, by material of high mobility electrophoresing just behind the dye front. This was overcome, either by raising the concentration of SDS in the sample buffer to 5% w/v, or precipitation of the sample with 10 volumes of acetone:ethanol (1:1). After precipitation the sample was collected by centrifugation, the pellet dried, and dissolved in sample buffer of the usual composition. Samples were loaded into the slots using a Hamilton syringe.

The slab gels described earlier give a 10% top to 15% bottom exponential gradient. However the technique could be adapted to give 10% left to 15% right horizontal exponential gradient of acrylamide. For this the gel cassette was constructed with one of the side spacers sealing 3.5 cm from the top of the gel cassette and the corresponding side left open. By standing the cassette with the open side upwards, a new cassette was formed with spacers across the bottom, top and opposite side. A 10-15% SDS-polyacrylamide slab gel could be poured as described earlier in this section into this cassette until 1 cm from the top (originally the open side) of the cassette. After polymerisation the top spacer could be removed and resealed along its original side with agar. After that electrophoresis could proceed as described earlier.

(b) Staining gels.

After electrophoresis all gels were fixed in 1:2:7 acetic acid: methanol:water for 20 mins at 37°C in a shaking water bath. The gels were then stained with Coomassie brilliant blue R (0.25%) in 7.5% v/v acetic acid, 50% v/v methanol for 20 mins at 37°C, and destained in 7% v/v acetic acid, 10% v/v methanol by diffusion at 37°C. The time taken for destaining could be accelerated by the addition of white wool or white sponge to the destaining solution, and reduced the time taken for destaining from 12-18 hours to 4-7 hours. After some time Coomassie brilliant blue R was withdrawn from the market and replaced without any noticeable differences by Kenacid blue (BDH). It was found to be more sensitive than Amido black 10b, or PAGE blue 83 (BDH). Lipoproteins were stained by a similar procedure in which the stain was Sudan Red IV.

Gels were photographed before drying. Drying of the gels was by heat evacuation onto 3MM Whatman filter paper using a Hoeffer gel dryer, model SE 540, with cellophane film over the top of the gel. The heating time was set at 30 mins and the dried down gel left to cool under vacuum. In order to reduce cracking in 15 or 20% acrylamide gels, gels could be soaked in 20% v/v propan-2-ol or 1% v/v glycerol to shrink and stabilise the gel. Also cracking of the gel could be avoided by raising the prepolymerised polyacrylamide concentration to 1% in the separating gel.

(c) Detection of radioactivity in polyacrylamide gels

Two basic methods have been used for the detection of ^{14}C and ^{3}H radioactivity in polyacrylamide gels; autoradiography of the dried gel, and liquid scintillation counting of gel slices.

(i) Gel slicing. Tracks to be sliced were excised from the stained and destained gel or from unfixed gels as described in the relevant

chapter, and were no more than 1 cm wide. Gels were sliced using a Mickle Gel Slicer (Mickle Engineering, Gomshall, Surrey) into 1 mm slices, the gel being kept moist by immersion in water. The slices were then dropped into 0.25 ml of NCS tissue solubiliser (New England Nuclear) which had been diluted with water (solubiliser:water 9:1) and incubated at 65°C for 90 min in capped vials. 2.5 ml of toluene based scintillant was added when the samples had cooled. Scintillation counting was performed in a Searle liquid scintillation counter, using the "variable quench" program, which assessed the degree of quench and presented the results as disintegrations per minute.

(ii) Autoradiography. Dried gels were autoradiographed in light-tight holders using Agfa Curix RP1 films. The X-ray film was developed by immersion in Agfa G150 developer (dilution 1:5 with water), development stopped in 5% v/v acetic acid for five minutes, and fixed in Agfa G334 (diluted 1:4) for five minutes. The fixative solution contained 2.5% Aditan to harden the X-ray film.

The image on the X-ray film could be darkened by bleaching and redevelopment of the autoradiograph when the image on the autoradiograph was very faint. The method is that of Zelger (1970). X-ray films were immersed in 200 mls of 7.6% w/v copper sulphate, 3.3% v/v acetic acid, 0.66% w/v potassium iodide and 7.6% v/v ammonia (specific gravity 0.88) until the black had turned yellow. The solution was thoroughly washed off and the autoradiograph immersed in 0.4% w/v silver nitrate, 1.6% w/v sodium acetate until the X-ray had turned black. This method increased the density of the image, but also darkened the background.

The time for autoradiography could be reduced by exposing X-ray films to gels impregnated with scintillant. The method is that of Chamberlain (1979). Gels were fixed, washed thoroughly with water, and

immersed in 10 volumes of 1 M sodium salicylate for 60 min. Care was taken to ensure that the gel was at neutral pH as salicylic acid is highly water insoluble. The gel was dried down, exposed to X-ray films at -70°C, and developed as described earlier after warming the holder to room temperature.

2.2.6. Immune Replicas

This technique (Towbin et al. 1979) permits the detection and measurement of very small amounts of a given protein, if specific antisera are available. Following electrophoretic transfer of the protein to cellulose nitrate sheets it is reacted with antibody and the immune complex decorated with ^{125}I-labelled protein A. The replica is then autoradiographed. The technique was used to measure F_1-ATPase, using an antiserum raised in rabbits against beef-heart mitochondrial F_1. This serum was the gift of Dr. D.K. Apps.

(a) Electrophoretic transfer of proteins. Polyacrylamide gel electrophoresis was carried out as described earlier. Transfer of proteins onto cellulose nitrate sheets (approx. 15 x 15 cm) was exactly as described by Towbin et al. (1979).

(b) Immune replica of cellulose nitrate sheets. After transfer of proteins the cellulose nitrate sheet was placed carefully, protein binding face upwards, in 50 mls of buffer containing 30 mg/ml BSA, 20 mM Tris-HCl pH 7.2, 0.9% w/v sodium chloride, 0.05% sodium azide and agitated for 60 minutes in this solution. This was removed and the replica washed for 90 minutes with antiserum solution. This contained 1 ml of antiserum, diluted with 50 mls of a buffer containing 5% v/v horse serum (previously inactivated by heating at 50°C for 30 mins, filtering through cellulose nitrate), 25 mg/ml BSA, 0.5 mg/ml sodium azide, 20 mM Tris HCl pH 7.2. The antibody solution was removed and

the cellulose nitrate sheet washed five times with 100 mls of 0.9% sodium chloride, 20 mM Tris HCl pH 7.2 (washing buffer) for 10 min each to thoroughly remove unbound antibody. Then the cellulose nitrate sheet was incubated for 60 mins in ^{125}I-labelled protein A (0.5 µCi/ml in 0.9% sodium chloride, 20 mM Tris-HCl pH 7.2). The protein A solution was then discarded and the cellulose nitrate sheet washed five times in 100 ml of washing buffer. The cellulose nitrate sheet was then dried and autoradiographed at room temperature for 1-3 days.

(c) ^{125}I labelling of protein A. To 1 ml of a solution of Staphylococcus aureus protein A (Pharmacia, 1 mg/ml in 0.1 M sodium phosphate buffer pH 7.4) were added Na^{125}I (1 mCi) and chloramine T (6 µmol). After 10 min at 0^{o}C, 10 µmol of $Na_2S_2O_5$ was added to stop the reaction and the mixture passed through a 10 x 0.8 cm^2 column of Sephadex G-10, equilibrated with 0.1 M sodium phosphate pH 7.4. Fractions of approximately 0.2 ml were collected, and their radioactivity measured by counting 2 µl samples in a Wilj gamma-counter. The final specific activity of the protein A was about 0.8 mCi/mg of protein.

2.2.7. Amino acid analysis

Amino acid analysis was performed on acid hydrolysates of proteins using the method described by Gardner (1981) on a Locarte IV autoanalyser except that elution times for the second and third buffers were changed to 44 and 100 mins respectively. Standard amino acids contained 26 nmoles each.

Calibration for tryptophan was performed using acid hydrolysates of glycyl tryptophan. 40 nmoles of glycyltryptophan, determined spectrophotometrically, were hydrolysed in 100 µl of mercaptoethane sulphonic acid (MESA) or 300 µl of 6M HCl in sealed evacuated tubes at 105^{o}C. Analysis of the HCl-hydrolysed sample was performed on the

hydrolysate dried in a vacuum dessicator over sodium hydroxide pellets and resuspended in sodium citrate pH 2.2. The MESA hydrolysate was neutralised with NaOH before analysis. Tryptophan could be detected in the MESA hydrolysate and a tryptophan degradation product, which eluted 8 minutes before tryptophan but with the same colour constant, could also be detected. Analysis of the tryptophan degradation product may be usable as a method of detecting tryptophan in HCl hydrolysed material.

2.2.8. Separation of dansyl amino acids on polyamide plates

Separation of dansyl amino acids on polyamide plates was by the method of Woods and Wang (1967) with modifications (Valentine, Ph.D. thesis, 1978). Double sided 15 x 15 cm polyamide plates (BDH) were cut into four 7.5 cm squares for general use. A cross was placed in one corner 1 cm from either edge, and dansyl amino acids were loaded onto the cross, using a microsyringe. Approximately 1 nmol of dansylated, acid hydrolysed protein was loaded onto one side, whilst a solution of standard dansyl amino acids was loaded onto the other.

Separation was by capillary action; in the first dimension using 1.5% v/v formic acid, until the solution front was 1 cm from the top of the plate. After drying the plate the second dimension was chromatographed, amino acids being separated in a dimension perpindicular to the first in a solution of 9:1 toluene:glacial acetic acid. After chromatography in this direction the plate was dried, inspected under ultraviolet light, and a drawing made. Then a third chromatographic separation was run, in the same direction as the second, using 30:20:1, butyl acetate:methanol:acetic acid. The plate was dried and the spots inspected under ultraviolet light.

Chapter 3

Effects of DCCD on chromaffin granule membrane

3.1 Introduction

Obtaining chromaffin granule membranes that are free from contamination by inner mitochondrial membrane is obviously of major importance in undertaking work on binding of DCCD to the membranes. The first part of this chapter deals with this aspect of the project, whilst the remainder deals with work on labelling of the membranes with ^{14}C-DCCD. Smith and Winkler (1967) produced a method for purification of chromaffin granule membranes on a large scale by differential centrifugation which was based upon earlier work (1966). Adrenal glands were obtained fresh from the slaughterhouse, the medullae were excised, homogenised in 0.3 M sucrose and subjected to low speed centrifugation, to remove nuclei and cell debris, and then high speed centrifugation to pellet a 'large granule' fraction. This was resuspended in 0.3 M sucrose, layered over 1.6 M sucrose, and ultracentrifuged to produce 'pure' granules as the pellet of the 1.6 M step gradient. All subsequent methods for purification have been based upon this method, and the initial three stages remain largely unchanged. Helle et al. (1971(a)) minced the medullae to ease the homogenisation, as the medulla is a 'tough' tissue. They also used a larger volume of 1.6 M sucrose in order to obtain a purer granule preparation. A further modification (Schneider, 1972) was to use a 1.8 M sucrose centrifugation step to obtain a pure granule suspension, and to introduce a further purification stage for granule membranes; these were obtained by lysing the granules (by freeze-thawing or osmotic shock), collecting the membranes, and layering over 1.0 M sucrose before further ultracentrifugation, thereby removing dense contaminants such as inner mitochondrial membranes. Phillips (1973) introduced the use of the impermeant zwitterionic buffer Hepes into the preparation procedure; this was found to improve the integrity of the granules. Subsequent purification methods have been based on the use of

different density gradient materials such as metrizamide (Morris and Schovanka, 1977), AG 6227 (Nordmann and Aunis, 1980) or percoll (von Grafenstein et al. 1980).

Contamination with inner mitochondrial membranes was assessed in the various preparations by a variety of marker enzymes. Smith and Winkler (1966) used fumarase, a protein of the mitochondrial matrix, as a marker for mitochondria, whilst Phillips (1973) used monoamine oxidase, a component of mitochondrial outer membranes. Schneider (1972) used succinate dehydrogenase whilst Helle et al. (1971(a) and Flatmark et al. (1971) used succinate oxidase. Use of fumarase is questionable as this only indicates the presence of intact mitochondria, whilst succinate oxidase activity depends upon the intactness of the mitochondrial electron transport chain. Smith and Winkler quote 2.1% contamination with mitochondria, whilst Phillips (1973) measured large contamination of granules with monoamine oxidase. Apps and Schatz (1979) used several different marker enzymes to assess mitochondrial contamination of their preparation of chromaffin granule membranes. The most pessimistic estimate (1.3%) was calculated on the basis of cytochrome oxidase activity. This enzyme is probably the most stable of the mitochondrial respiratory enzymes, and is certainly the most active and easy to assay; it is therefore a very suitable marker. Both Helle et al. and Schneider could not detect the presence of any mitochondria in their preparations. The markers for chromaffin granule membranes used here were either dopamine β-hydroxylase activity or the content of cytochrome b_{561} in the solution. Both are known to be membrane proteins of the chromaffin granule and whilst dopamine β-hydroxylase is extremely difficult to assay accurately, cytochrome b_{561} content presents a stable, though less sensitive, marker for the granule membrane. Purification of granule membranes in this work is by a modified method of Schneider or Apps and

Schatz and is as described in Materials and Methods (Chapter 2).

Several studies have been made of the labelling of various mitochondrial components with ^{14}C-DCCD. Cattell et al. (1971, Figure 2a) found at least two labelled bands in intact ox heart mitochondria, at a concentration of DCCD required to inhibit 95% of the ATPase activity, although a major proportion of the label is located in a band of low molecular weight, as seen by SDS gel electrophoresis. Steckhoven et al. (1972) showed that at low concentrations of DCCD only one band of protein was labelled whilst at higher concentrations several bands could be identified from electrophoretograms of purified beef heart mitochondrial ATPase. In E. coli plasma membranes, at least five different proteins can be labelled with ^{14}C-DCCD (Fillingame, 1975), whilst only one protein was covalently labelled in chloroplasts (Sigrist-Nelson and Azzi, 1979). It appears that the specific binding of DCCD to the low molecular weight subunit of the ATPase is only manifested at low concentrations, whilst at higher concentrations the reactivity of the carbodiimide leads to nonspecific covalent modification of many membrane proteins. Among the other components of the mitochondrial membranes known to be covalently modified by DCCD is the β-subunit of the ATPase (Pougeios et al., 1980) and subunit III of the cytochrome c oxidase complex (Casey et al., 1980).

3.2. Results

3.2.1. Purification of chromaffin granule membranes.

Chromaffin granule membranes were purified as described in Materials and Methods. Cytochrome c oxidase, dopamine β-hydroxylase, succinate dehydrogenase activities, cytochrome b_{561} (type I assay) and protein (Hartree-Lowry assay) contents were determined as described in Chapter 2. Adrenal mitochondrial membranes were purified as described in Chapter 2.

Stage in preparation	Protein mg ml^{-1}	Cytochrome oxidase nmol min^{-1} ml^{-1}	Cytochrome b_{561} µg ml^{-1}	Fraction volume ml
4K supernatant	6.49	713	52	894
14K supernatant	3.58	33	7.3	859
14K pellet	25.8	5413	403.2	99
1.7 M supernatant	2.3	95	13.8	935
1.7 M layer	20.9	11224	125.4	31
1.7 M solution	2.6	192	32.5	272
1.7 M pellet	11.92	248	214.6	75
Chromaffin lysate	3.3	0	0	200
Chromaffin pellet	16.6	945	1128	14.3
Mitochondria lysate	2.15	8.5	0	200.5
Mitochondria pellet	4	17128	99	17.6
Chromaffin 1.0 M supernatant	2.2	35	2.48	14.5
" 1.0 M layer	11	371	924	8.8
" 1.0 M solution	1.3	138	0.46	44
" 1.0 M pellet	3.3	928	0.18	8.6
Mitochondria 1.0 M supernatant	3.53	0	10.59	14.4
" 1.0 M layer	7.52	208	52	3.7
" 1.0 M solution	4.1	138	20.5	44
" 1.0 M pellet	10.9	14715	21.8	29

Table 3.1.(a). Marker Enzyme Activities of a Typical Preparation Procedure of all fractions.

The markers used are cytochrome b_{561} for chromaffin granule membranes and cytochrome oxidase for inner mitochondrial membranes. Assay methods are described in Chapter 3.2.1. and purification procedure in Chapter 2.2.1.

FRACTION	Cytochrome oxidase nmol min^{-1} mg $protein^{-1}$	Cytochrome b_{561} µg mg $protein^{-1}$	Succinate dehydrogenase nmol min^{-1} mg $protein^{-1}$
4K SN	109.86	8	34
1.7 M layer	537	6	170
Chromaffin 1.0 M layer	33	84	10
Mitochondria 1.0 M pellet	1350	2	416

Table 3.1 (b) Specific activities of important fractions from the purification procedure.

Activities calculated from Table 3.1.(a) and enzymes assayed as described in Chapter 2.

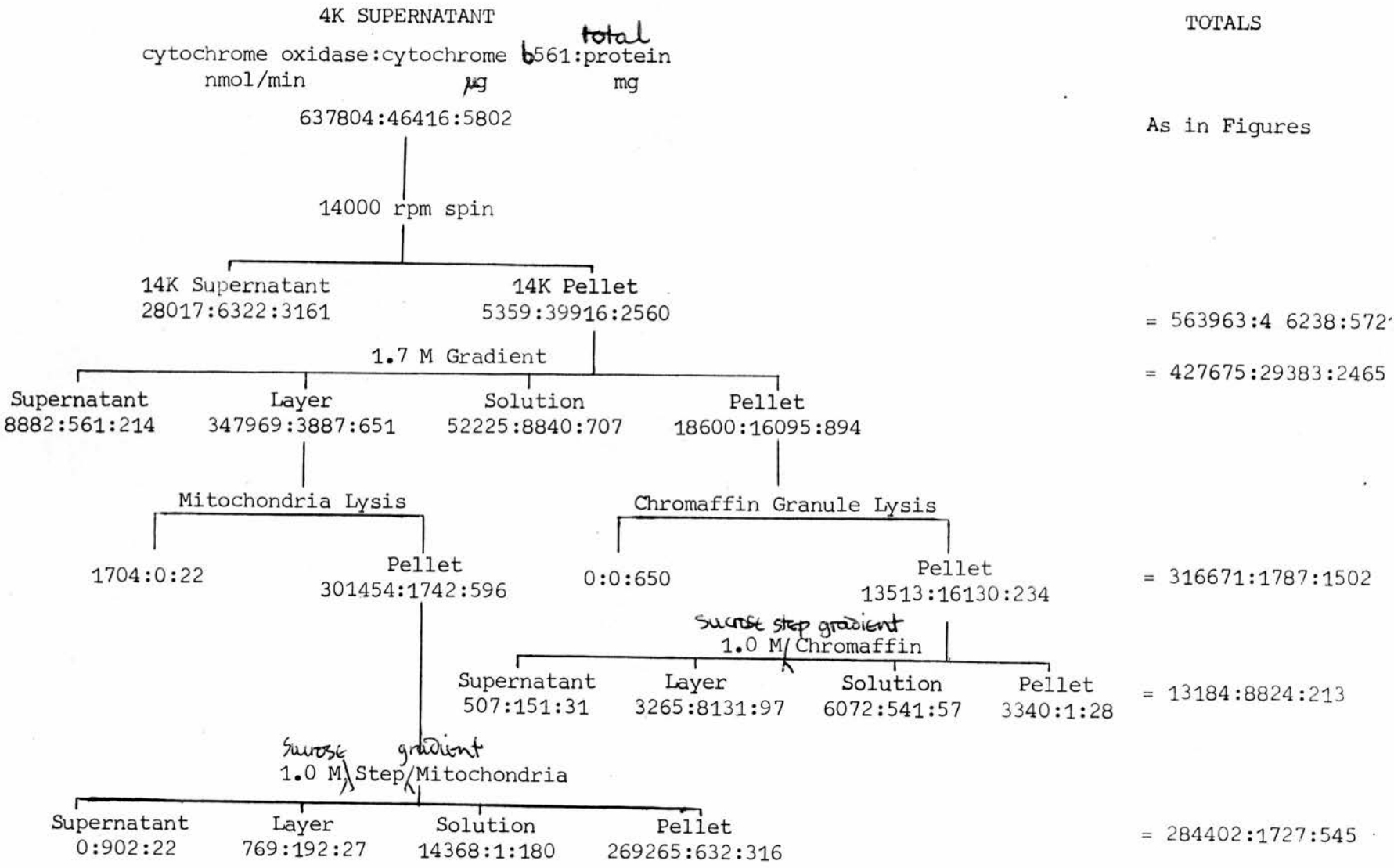


Figure 3.1. Flow diagram of preparation procedure giving Total activities for both cytochrome oxidase and cytochrome b... in all possible fractions. Protein estimations

Results. Small samples of solution were taken at each stage of the preparation procedure and the total volumes of each fraction noted. Results are presented in Table 3.1(a) and (b) and Figure 3.1. From Table 1(b) cytochrome c oxidase and succinate dehydrogenase activities appear to parallel each other. This parallel copurification of these activities suggests that both of these activities are part of the same organelle fraction. The possibility of cytochrome c oxidase activities being associated with chromaffin granules is thus much diminished (Flatmark et al. 1972). This result suggests that the residual activity of cytochrome oxidase is simply a function of some mitochondrial impurities in the fraction, probably due to membrane aggregation during the preparation of the chromaffin granules. There is an approximately ten-fold increase in specific content of the chromaffin granule membranes using cytochrome b_{561} as a marker. This may well be an underestimate of the actual enrichment as Figure 3.1 demonstrates large losses in the total cytochrome content at both sucrose density gradient steps. The reason for this is somewhat difficult to explain but any osmotic shock undergone during these steps may have lead to release of iron from the heme group of these fractions. Another explanation is that the extensive dialysis needed to remove catecholamines before assaying for cytochrome b_{561} content may have resulted in loss of heme or iron.

The final contamination of the chromaffin granule membranes by mitochondria is about 2.5%, calculated from the specific activities in both mitochondrial and chromaffin granule membranes for both succinate dehydrogenase and cytochrome c oxidase. This low level of contamination differs only slightly from results of Apps and Schatz (1979), which may be accounted for by the use of 1.8 M sucrose instead of 1.7 M sucrose in the preparation procedure published here. However these results differ from those of Schneider (1972), and others, who were unable to

find any mitochondrial contamination in their preparation. This may well be due to the type and sensitivity of the marker enzyme assay used. Of the mitochondrial assays used the most sensitive and stable complex to use as a marker is the cytochrome oxidase, which only requires reduced cytochrome c and oxygen to function. However, succinate oxidase requires an intact electron transport chain to function, which may not exist after several ultracentrifugation steps. Thus the cytochrome oxidase activities proposed by Helle et al. (1971(b)) as a real function of chromaffin granule membranes may well be due to mitochondrial contamination, as these authors used succinate oxidase as a mitochondrial marker. The possibility that very low levels of cytochrome c oxidase activity exists in chromaffin granule membranes cannot be entirely ruled out from this work and immunological techniques using anti-cytochrome c oxidase antibodies may well shed more light on the presence of cytochrome c oxidase in the chromaffin granule membrane.

It has been shown here that for general use chromaffin granule membranes of high purity can be obtained. Calculations based upon immune replica using anti-beef F_1 antibodies (see Chapter 5) give the ATPase as 0.6% of the total membrane protein (weight for weight) and the mitochondrial F_1 as 10.3% of the total protein. If this figure is correct then 2.5% contamination of chromaffin granule membranes by adrenal mitochondria gives a value of about 33% for the amount of contamination by mitochondrial F_1 of chromaffin granules. If this were the case then it might be expected to observe significant inhibition of membrane ^{A}TPase activity by oligomycin or efrapeptin, but this has not been demonstrated (Apps and Glover, 1978). The explanation of this observation may be that adrenal mitochondria aggregating with chromaffin granules are oligomycin insensitive; that chromaffin granule ATPase in the membranes is much more active than the isolated enzyme; that there

is more than one ATPase in the membrane, or that some of the cytochrome oxidase activity is real. To test these hypotheses demand more experimental work.

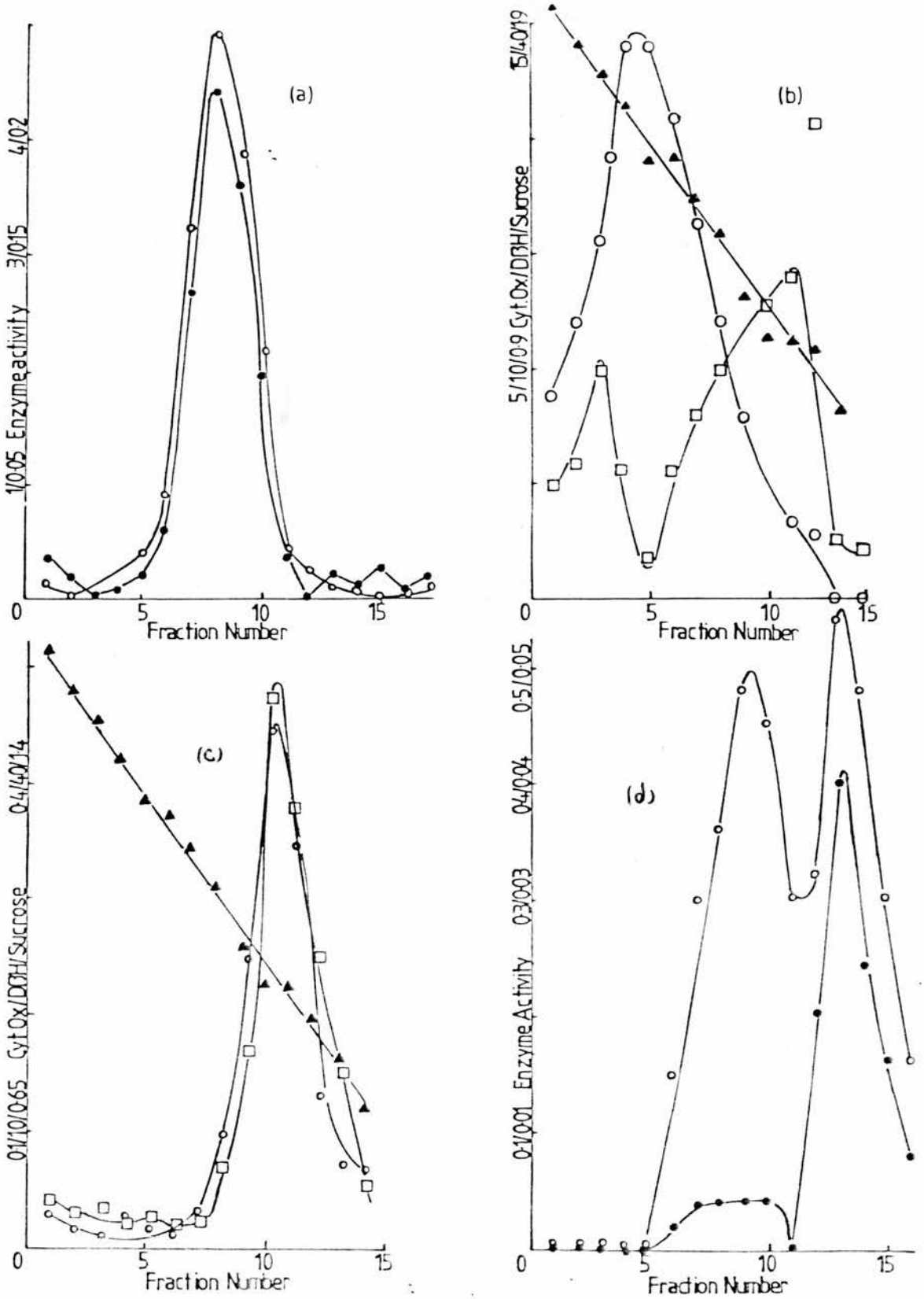
In order to ascertain whether the observed cytochrome oxidase levels were due to contaminants, some minor modifications were made to the standard preparation procedure. Further purification of granules and mitochondria on continuous sucrose gradients was also attempted. The problem with contamination may well be due to an effect of membrane aggregation, possibly during the centrifugation at 14000 r.p.m. in which mitochondria and chromaffin granules are pelleted together. This may be further aggravated by the homogenisation that follows, in order to re-suspend the pellet. One way of overcoming this problem is to add membrane disaggregants to the preparation procedure. One of the well known aggregants of membrane is Ca^{2+} especially as chromaffin granules are secretory vesicles and calcium ions have been shown to be important in the 'stimulus-secretion' event. It was decided to investigate the effect of including EDTA, a calcium chelator, in the buffers.

The theory that membranes aggregate during the 14000 r.p.m. centrifugation step is also suggested by another result. During the preparation of chromaffin granule ghosts, a wash with 0.3 M sucrose of the 14000 r.p.m. pellet, removes the brown layer leaving only a pink pellet. Presuspension and rewashing yields quite pure granules even though a sucrose density gradient had not been employed (Phillips and Allison, 1978).

Results. Chromaffin granule membranes were prepared by the method previously described except that all solutions also contained 1 mM EDTA in the mixture. Cytochrome oxidase and succinate dehydrogenase activities were measured as described previously. The final activities in the membranes purified by this method were 24 nmol min^{-1} mg^{-1} for cytochrome oxidase and 7 nmol min^{-1} mg^{-1} for succinate dehydrogenase. Since cytochrome

Figure 3.2. Continuous 0.5-1.5 M sucrose density gradient of various membrane fractions by marker enzyme assays

(a) Purified mitochondria, cytochrome oxidase (o) and succinate dehydrogenase (●)
(d) Chromaffin granule membranes, symbols as in (a)
(c) Chromaffin granule membranes from purified fraction of continuous 0.5-1.5 M sucrose on second sucrose gradient, (o) cytochrome oxidase, (□) dopamine β hydroxylase, (▲) sucrose concentration.
(b) As in (c) except before second continuous gradient granule membranes subjected to a mild sonication.



oxidase and succinate dehydrogenase activities both decrease in parallel, this simply lends weight to the argument that the presence of cytochrome oxidase activity is an artefact due to mitochondrial membrane contamination. It seems that there is some membrane aggregation due to the presence of divalent cations.

Further purification by use of continuous sucrose density gradient centrifugation of EDTA-treated membranes was also attempted. The 12.8 ml gradient (0.5-1.5 M sucrose) were poured as described in Chapter 2. Gradients of both purified chromaffin granule membranes (1.0 M layer) and mitochondrial membranes (1.0 M pellet) were both centrifuged. The gradients were divided into 0.8 ml fractions. Mitochondrial and chromaffin granule rich fractions are distinctly separated by this method, pink chromaffin granule membranes equilibrating at 0.8 M sucrose and brown mitochondrial membranes at 1.2 M sucrose. These results agree closely with those published by several other groups (Winkler 1966; Phillips, 1973; Helle et al. 1971(b)). Fractions from the gradient were assayed for dopamine β-hydroxylase, succinate dehydrogenase, and cytochrome c oxidase and presented in Figures 3.2(a)-(d).

From these profiles several conclusions can be drawn. The distribution of cytochrome oxidase and succinate dehydrogenase are clearly similar in purified mitochondria. Equilibrium centrifugation of chromaffin granule membranes reduced the specific activity of cytochrome oxidase by 50% but the residual cytochrome oxidase activity was not further reduced by a second isopycnic centrifugation. When the chromaffin granule membranes were subjected to mild sonication before centrifugation, further separation of cytochrome oxidase and dopamine β-hydroxylase activity was achieved. However on these gradients the membranes were spread over a much larger area and did not band as tightly as the unsonicated material. Unfortunately when this technique of sonication was applied to membranes routinely prepared prior to centrifugation over 1.0 M sucrose it was found

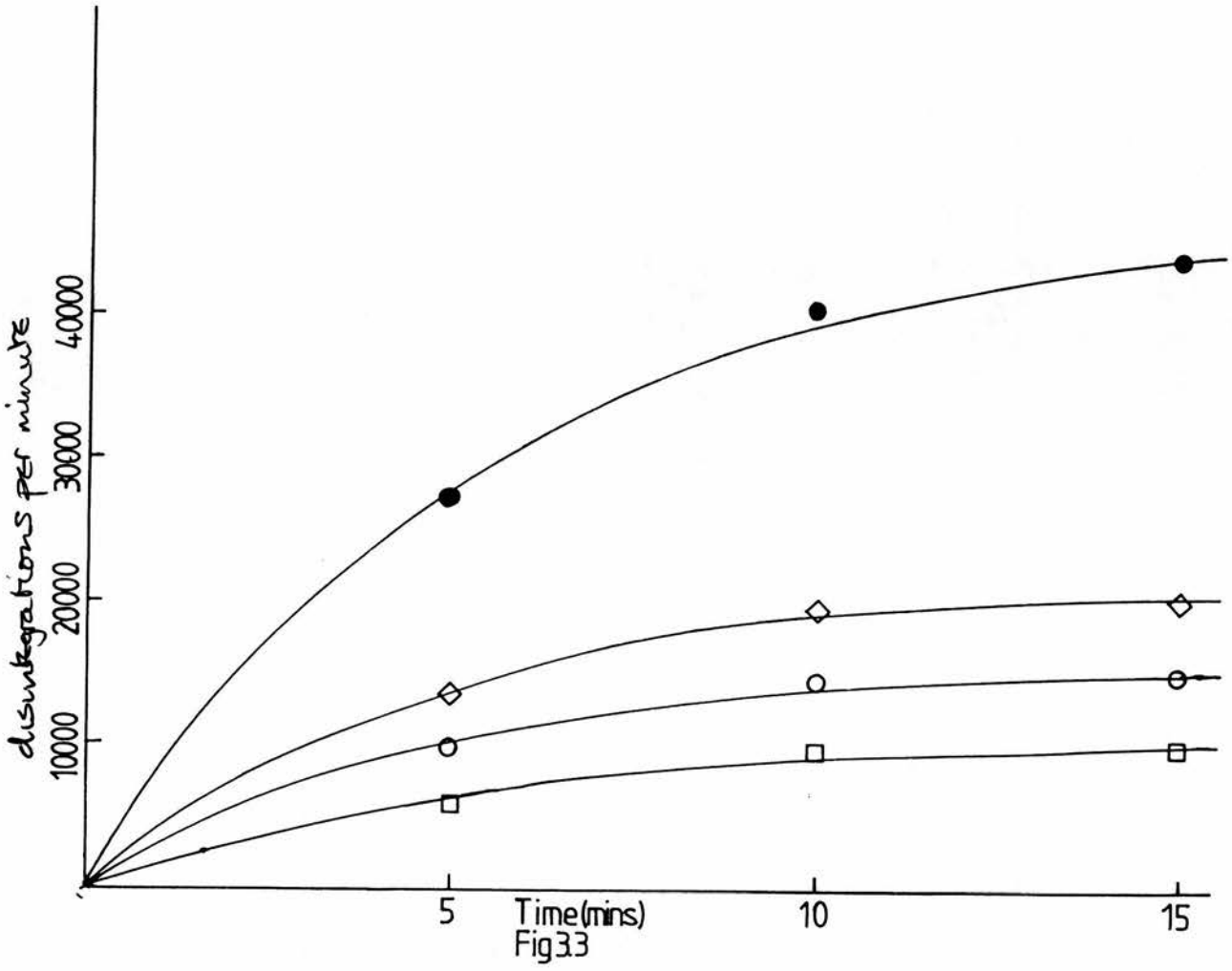


Figure 3.3. Binding of ^{14}C-DCCD by chromaffin granule membranes

Binding was assayed as described in the text at the following DCCD concentrations, □ 5 µmolar; ○, 7.5 µM; ◇ 10 µM; ●, 20 µM.

that the resultant membranes did not become concentrated at the 1.0 M interface but were instead spread throughout a large volume and thus sonication could not be used in routine preparations.

Several conclusions can be drawn from work on purification of chromaffin granule membranes. Firstly the standard purification method achieves membranes of high purity although there appears to be some contamination by mitochondrial membranes. Secondly cytochrome oxidase activity can be completely separated from dopamine β-hydroxylase activity, suggesting that the cytochrome oxidase activity observed by Helle et al. (1971(b)) is simply mitochondrial contamination. For general work, that is most of the work described in the thesis chromaffin granule membranes were purified until the 1.0 M sucrose step was reached.

3.2.2. Labelling of chromaffin granule membranes with ^{14}C-DCCD

In this section the evidence for the presence of a DCCD-reactive protein in chromaffin granule membranes is considered, and evidence that DCCD labels part of the ATPase complex is presented.

In a preliminary experiment, freshly prepared chromaffin granule membranes were incubated at 4°C (Hepes buffer, 1 mg ml^{-1} protein) with 5, 7.5, 10 and 20 μM ^{14}C-DCCD. An attempt was made to assay for incorporation of DCCD into the one protein by counting radioactivity incorporated into the membranes, by simply taking 100 μg samples at various times, filtering onto cellulose nitrate filters (0.45 μm) washing and counting for radioactivity in scintillant containing 33% Triton X-100. Over a 15 min period (see Fig. 3.3) 90-100% of the radioactivity became associated with the membranes, and the system showed no saturation at well above the maximal levels of DCCD required to modify all of the available DCCD reactive protein. It is noteworthy, that owing to the ease of hydration of the carbodiimide that large excess has to be added in order to find any

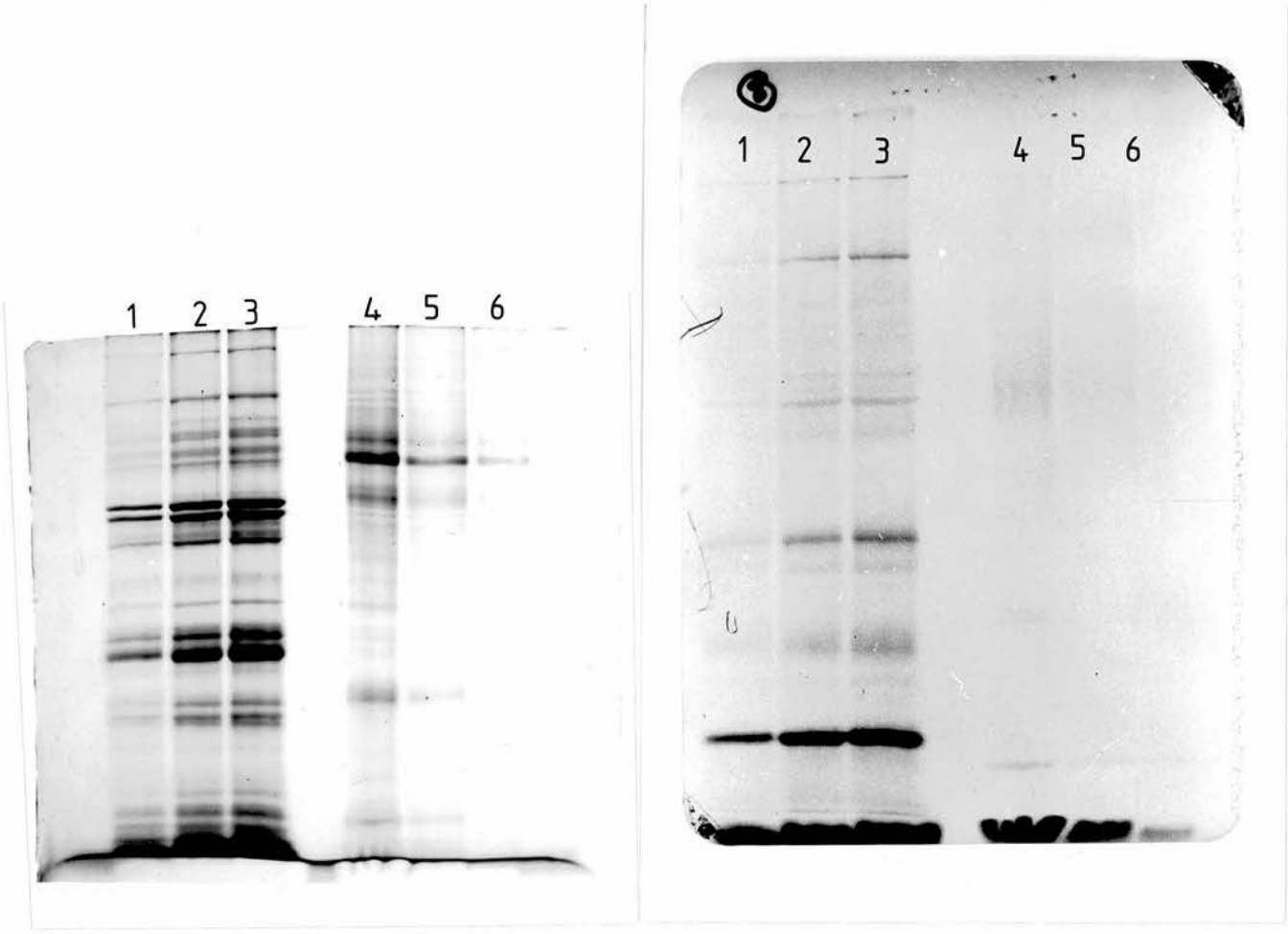


Figure 3.4. Binding of ^{14}C-DCCD to chromaffin granule membrane proteins

(a) 10-15% exponential SDS polyacrylamide gels stained with Coomassie for protein Tracks 1-3 mitochondria labelled with ^{14}C-DCCD; Tracks 4-6 chromaffin granule membranes labelled with ^{14}C-DCCD.

(b) Autoradiograph of (a) not on the same scale.

reactive proteins. Also with DCCD both the carbodiimide and the dicyclohexylurea, the hydration product, are hydrophobic and will probably partition into the membrane. DCCD is also not necessarily specific in its reactivity for any particular protein and various membrane components may bind DCCD. Thus, covalent modification of any single protein may not be observable by this method. The method adopted to investigate incorporation of DCCD into a specific protein was that of SDS polyacrylamide gel electrophoresis, followed by autoradiography.

Freshly prepared chromaffin granule and adrenal mitochondrial membranes were suspended in 10 mM Hepes, 1 mg protein ml^{-1}, $4^{o}C$, and a final concentration of 20 μM ^{14}C-DCCD added to the incubation medium, which was then left for 16 h. Membranes were then dissolved in SDS sample buffer and subjected to polyacrylamide gel electrophoresis on 10-15% gradients and autoradiography as described in Chapter 2. It was found that eight weeks were needed in order to obtain the amount of darkening of the autoradiograph seen in Figure 3.4(b). Figures 3.4(a) and (b) show the result of this experiment. The chromaffin granule membrane appears to contain a low molecular weight polypeptide which is labelled by ^{14}C-DCCD. This polypeptide is labelled much more weakly than the corresponding band in the mitochondrial electrophoretogram. This could either be due to a much lower concentration of the polypeptide in chromaffin granule membranes, or due to inaccessibility of some of the binding sites in the granule membrane for DCCD. The labelled low molecular weight band appears to migrate with a slightly higher electrophoretic mobility than the mitochondrial polypeptide. The question of whether this difference is the result of an artefact produced by the gel electrophoresis system due to mitochondrial contamination of the chromaffin granule membrane remains unanswered by this method. The possibility that the level of ATPase in chromaffin granules is artefactual

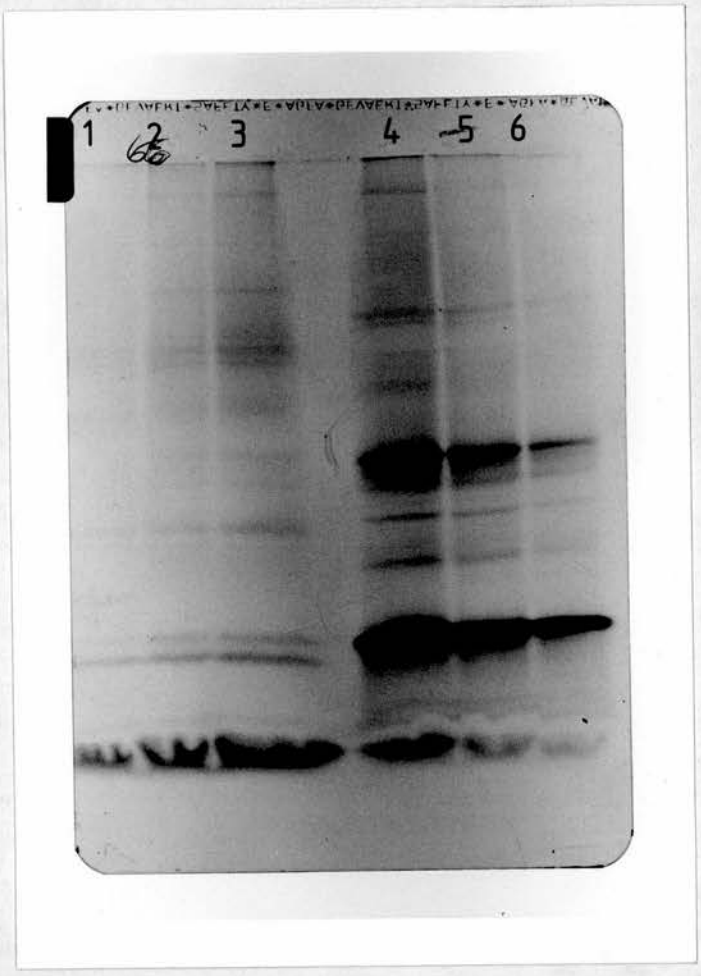


Figure 3.5. Migration of DCCD-reactive protein on 10-15% exponential SDS-polyacrylamide gels

Autoradiograph of 10-15% exponential SDS polyacrylamide gel showing, left, chromaffin granule membranes ^{14}C-DCCD labelled to which a small amount of mitochondria had been added, and right, ^{14}C-DCCD labelled mitochondrial membranes. Time of autoradiography was about fourteen weeks.

is raised by the similarity in structure of the soluble fractions of mitochondrial and chromaffin granule ATPases (Apps and Schatz 1979). Differences in the lipid composition of the granule and mitochondrial membranes could seriously affect the gel pattern, if the lipid:protein ratio is as large as it is in chromaffin granules. Also, the fact that a single charge difference on the protein can affect its electrophoretic mobility in SDS gels suggests caution in accepting this result. A further cause of doubt is the low amount of incorporation of ^{14}C DCCD into the labelled band in the chromaffin granule membrane. If the two proteins are in fact different this strengthens the overall impression that the proton translocating ATPase of chromaffin granules is different but similar to the mitochondrial ATPase.

In order to verify that the two proteins are different a slight modification of the initial experiment was tried. A sample of 90 µl of labelled chromaffin granule membranes was mixed with 10 µl of mitochondrial membranes labelled as previously described. The proteins were then dissolved in SDS and subjected to electrophoresis and autoradiography as previously described. The autoradiograph can be seen in Figure 3.5. From this autoradiograph two bands of low molecular weight can be seen in the chromaffin granule membranes, whilst in mitochondria only one band of low molecular weight can be seen. One of the bands appears to have the same mobility as the labelled mitochondrial protein whilst the other appears to migrate slightly faster, comigrating with the band labelled in chromaffin granule membranes.

This conclusively shows that a low molecular weight protein in chromaffin granule membranes is covalently modified by ^{14}C-DCCD and that this protein has a different electrophoretic mobility to the low molecular weight band labelled in the adrenal mitochondria. From these gels it appears that the chromaffin granule band is of slightly lower

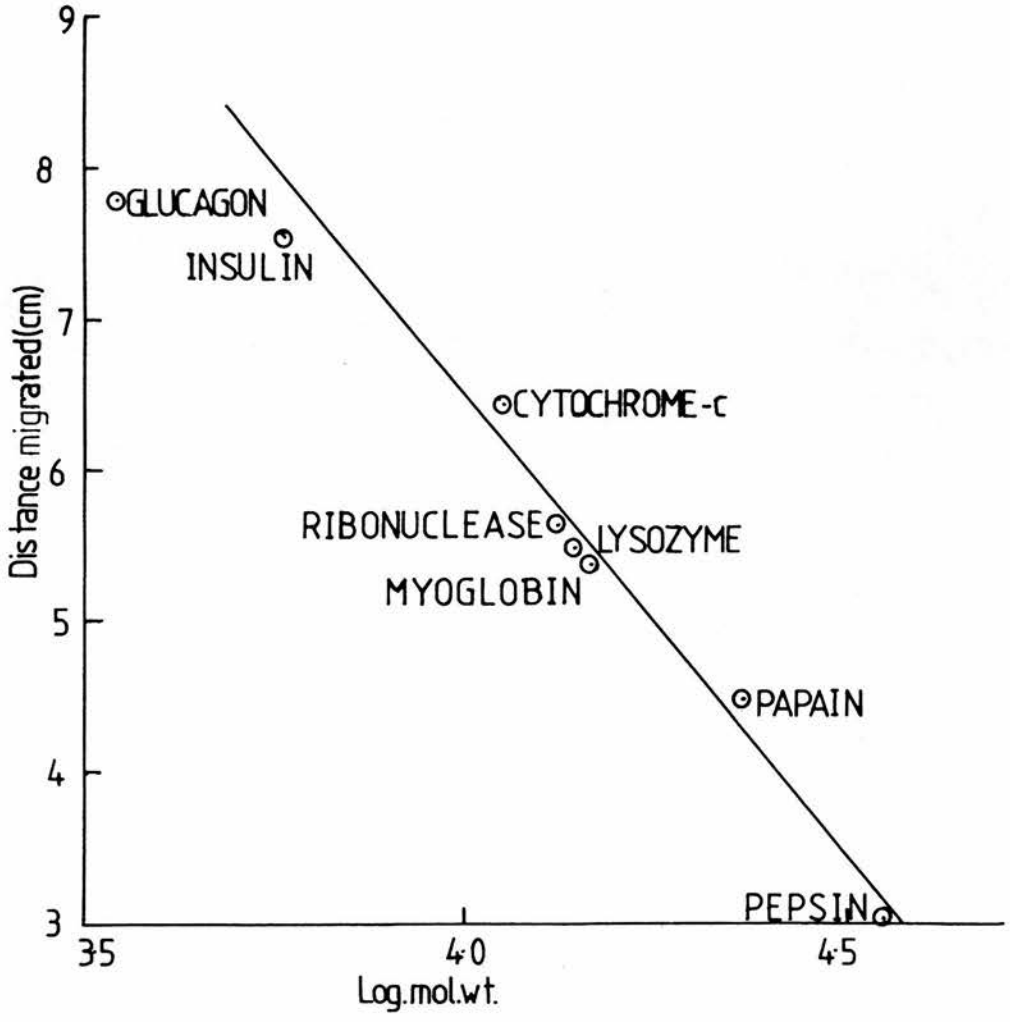


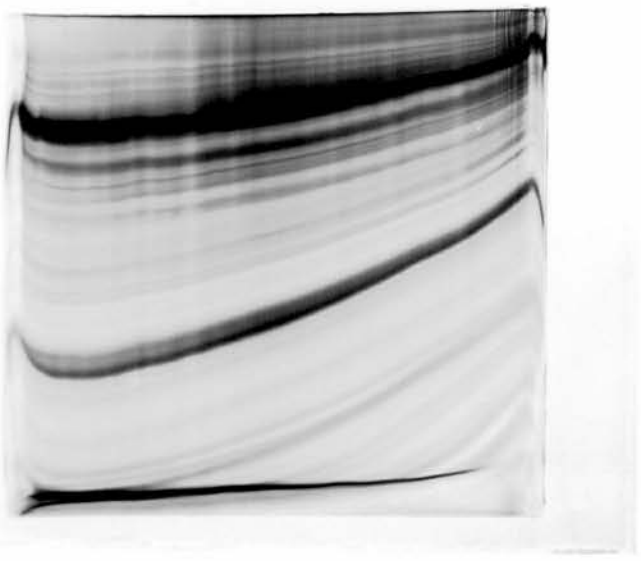
Figure 3.6. Molecular weight calibration of 15% acrylamide gels

15% SDS polyacrylamide gels were electrophoresed with various standard molecular weight markers and a plot of distance migrated against log molecular weight for proteins is presented here, as described in 3.2.3.1.

molecular weight than the corresponding band in mitochondria, although the small size and hydrophobic nature of these proteins may make molecular weight estimations by this technique unreliable.

3.2.3.1. Molecular weight determination. To obtain an estimate of the relative and actual molecular weights of the two proteins SDS polyacrylamide gel electrophoresis was employed. Since proteins of known molecular weight were to be compared with unknown labelled proteins a slab gel system was used. The most repeatable and reliable results previously published, linearly relating the logarithm of the molecular weight to the distance travelled were those of Weber and Osborn (1969) using a phosphate/SDS system in tube gels. It was decided to adapt their system for use in slab gels. However this was found to be inapplicable to slab gels, probably because of the high current and the large pH change which occurred in the system during electrophoresis. The system of Laemmli (1970) similar to the one described in Chapter 2 was used instead.

As the DCCD reactive proteins were of low molecular weight a 15% polyacrylamide gel should provide the best resolution in the molecular weight range of interest. Proteins of known Mr = 3,000-40,000 were electrophoresed on 15% polyacrylamide gels. A plot of log.molecular weight versus distance migrated is presented in Figure 3.6: a straight line can be drawn through all points with the exception of those for glucagon and cytochrome c. Other workers using a tube gel system where comparison of mobilities of proteins under electrophoresis required comparing different tubes calculated mobilities relative to the dye front. In the slab gel system the dye front ran parallel to the top of the separating gel so this sort of calculation was not necessary in order to obtain calibration curves. The mobility of cytochrome c also differed sharply from its expected value in the gel system used by Weber and



(a)

Figure 3.7. Horizontal 10-15% acrylamide gradient SDS polyacrylamide gel electrophoresis

(a) Horizontal 10-15% SDS polyacrylamide gel electrophoresis of chromaffin granule membranes labelled with ^{14}C-DCCD as described in the text.

(b) Autoradiograph of (a) x 2 approx.

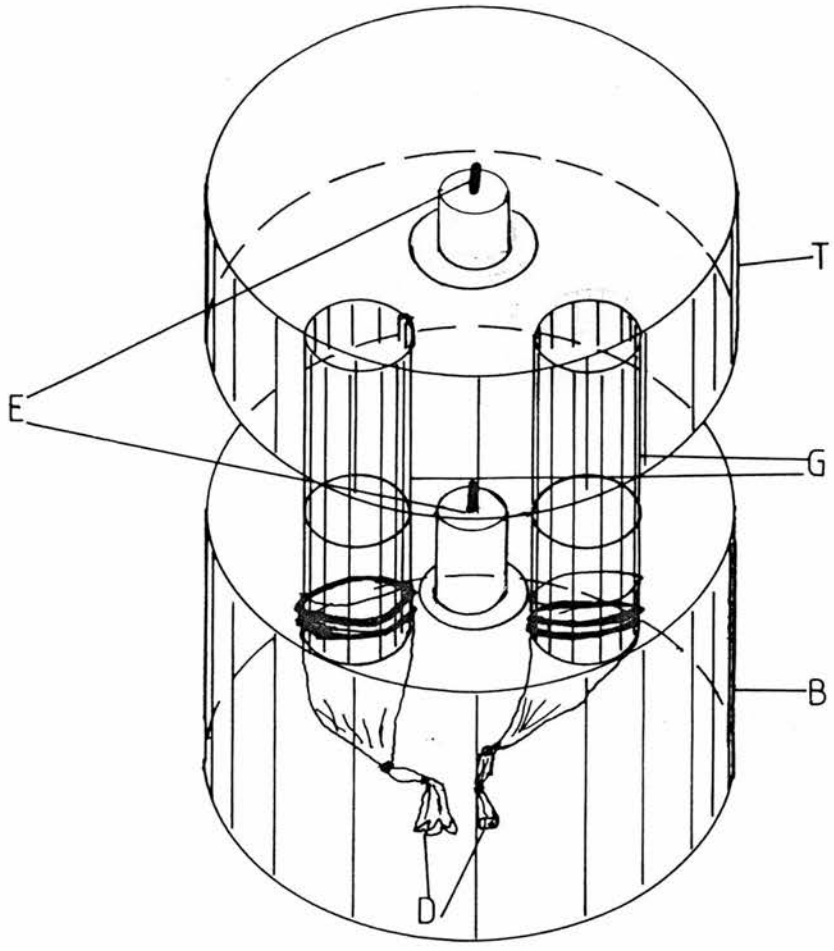


Figure 3.8. Electroelution apparatus

Apparatus used for partial purification of DCCD-reactive proteins from mitochondria and chromaffin granules from SDS gels. T = top tank; G = chamber for gels; B = bottom tank; D = dialysis bags; E = platinum electrodes.

Osborn, probably due to the basic nature of that protein.

An initial experiment, in which labelled chromaffin granule membranes and mitochondrial membranes were dissolved in SDS prior to gel electrophoresis in 15% gels to determine molecular weights was unsuccessful owing to low molecular weight material being retarded and distorting the protein pattern. Although this material was labelled it did not migrate as a protein and is probably lipid. A horizontal 10-15% exponential gel suggested that the problem could not be overcome by altering the percentage of acrylamide in the separating gel as lowering the percentage acrylamide lead to the DCCD-reactive protein electrophoresing with the dye front. The horizontal 10-15% gradient polyacrylamide gel is presented in Figure 3 7(a) and (b).

3.2.3.2. Electroelution. In order to separate the DCCD-reactive protein from the low molecular weight material, electroelution of the labelled band from SDS polyacrylamide gels was attempted. This system had previously been successfully used to obtain pure α and β subunits of the chromaffin granule ATPase (Apps and Schatz, 1979). The electroelution tank was made of perspex with platinum electrodes, and is shown diagramatically in Figure 3.8. Bands could be excised from 10-15% preparative exponential gels after staining with coomassie blue to locate the proteins, then loaded into the loading chamber of the electroelution apparatus and a potential of 200v, top negative, applied across the electrodes. The buffer tanks were filled with the standard Tris-glycine-SDS tank buffer. The proteins could be seen to concentrate in the bottom of the dialysis bags after electrophoresis out of the gel.

For a comparison of molecular weights, ^{14}C-DCCD labelled samples of both mitochondrial and chromaffin granule membranes were prepared, by the method described earlier in section 3.2.2. Proteins were dissolved in SDS sample buffer and electrophoresed, stained, and destained as

described in Chapter 2. The bands to be electroeluted were excised and loaded into chambers for electroelution, and electroeluted overnight. When concentrated protein could be seen in the dialysis bag electroelution was stopped, buffer removed from the top tank and gel chamber, the gel removed and protein withdrawn from the dialysis bags using a long pasteur pipette. The excised and eluted band was dried down with the remaining part of the gel and autoradiographed. Unfortunately, for both the chromaffin granule and mitochondrial proteins the eluted gel still contained the labelled material, whilst no labelled material could be seen in the 15% molecular weight gel. Apparently acid fixing of the protein resulted in its irreversible denaturation. This immediately suggests a similarity in the properties of the two proteins, as other proteins such as F_1-ATPase subunits and cytochrome b_{561} could be successfully electroeluted after acid fixation of the gel. A method of visualising the protein which did not remove the SDS from it was therefore sought.

Two methods of visualising the protein based upon precipitating the protein/detergent complex have been reported. One method involves the use of KCl to form insoluble potassium dodecyl sulphate (Hager & Burgess, 1980). The other relies on the salting out effect of high ionic concentrations to precipitate out the protein and used 4M sodium acetate (Higgins & Dahmus, 1979). These methods were tried and whilst other authors have reported considerable success in visualising proteins, both were found to be of poor sensitivity. 10-15% exponential gels of ^{14}C-DCCD labelled chromaffin granule and mitochondria were electrophoresed as described previously. After the tracker dye had reached the bottom of the separating gel, the electrophoresis was stopped and the gel was cut in half, and the two halves placed in 0.25 M KCl or 1 M sodium acetate. After 5 mins some protein bands were visible in the sodium acetate treated

Figure 3.9. Molecular weight determination of DCCD-reactive proteins by SDS polyacrylamide gel electrophoresis

15% polyacrylamide gel containing the following molecular weight markers:-

TRACKS 1. Glucagon. 2. Insulin. 3. Reduced chymotrypsin. 4. Mitochondrial electroeluted protein. 5. Chromaffin granule electro-eluted protein. 6. Pepsin. 7. Papain. Cytochrome c. = 9. Ribo-nuclease. = 10. Myoglobin. = 11 Lysozyme = 8.

Protein	Mol. wt.	log mol. wt.	Distance travelled cm
Glucagon	3485	3.541	12.6
Insulin	5734	3.758	12.1
Chymotrypsin (reduced)	11 + 13	4.041 + 4.114	9.9 + 8.3
Mitochondrial elute	15876	4.20	7.2
Chromaffin elute	14867	4.17	7.6
Papain	22960	4.361	6.0
Pepsin	34990	4.544	3.0
Myoglobin	17217	4.236	7.8
Cytochrome c	11700	4.068	8.0
Lysozyme	14300	4.155	7.9
Ribonuclease	13700	4.137	8.0

Table 3.2. Distance travelled versus molecular weight for some proteins of known molecular weight and determined from extrapolation of Fig 3.9 for the mitochondrial and chromaffin granule electroelute.

gel as either opaque bands (dopamine β-hydroxylase) or clear bands (cytochrome b_{561}) when compared to the background whilst nothing could be visualised with KCl. Lipid and SDS, concentrated at the front, appeared as an extremely opaque white band. Unfortunately only the major bands could be seen and the DCCD-labelled proteins could not. However since the object was simply to separate low molecular weight proteins from lipid, and as the protein was labelled with ^{14}C-DCCD, a wide strip which included the labelled band was excised. This was then loaded into the electroelution tank and eluted as previously described. The eluted material was collected from the dialysis bag, dissolved in pH 6.8 sample buffer and electrophoresed on the 15% gel used for molecular weight determination. The excised band of the original gel was fixed and stained before autoradiography as was the 15% gel. The 15% gel can be seen in Figure 3.9. The mobility of the stained bands from the dried down gel could be taken and compared to the distance migrated by the radioactive bands in the autoradiograph. The results can be seen in Table 3.2. The value reported here of 15800 for the molecular weight of the beef heart mitochondrial DCCD reactive-protein is rather high compared to those previously reported of 13500 (Steckhoven et al. 1972), 10000 (Cattell et al. 1971) and 8800 (Sebald et al., 1979a). The first two figures reported were determined by gel electrophoresis, whilst the third is obtained from sequence analysis of the protein. Two reasons could explain this difference; an artefact of the gel system or aggregation of the protein into dimers. Since these proteins are known to be very hydrophobic they may electrophorese unusually. Also SDS gel electrophoresis of low molecular weight polypeptides for molecular weight determination is notoriously inaccurate. However this method clearly shows the chromaffin granule DCCD binding-protein (Mr = 14800) migrates faster than the mitochondrial protein.

Final concentration of constituents	1	2	3	4	5	6
100 mM Hepes-NaOH pH 7.4	+	–	+	+	+	+
100 mM Mes-NaOH pH 5.6	–	+	–	–	–	–
10 mM ATP-NaOH pH 7.4	–	–	+	+	+	+
5 mM $MgCl_2$	–	–	–	+	–	+
1 mM EDTA	–	–	–	–	+	+
10 µM ^{14}C-DCCD	+	+	+	+	+	+

Table 3.3. Media for incubation of chromaffin granule membranes with ^{14}C-DCCD.

The table shows the final concentration of constituents and the different media used. Results can be seen in Figure 3.10.

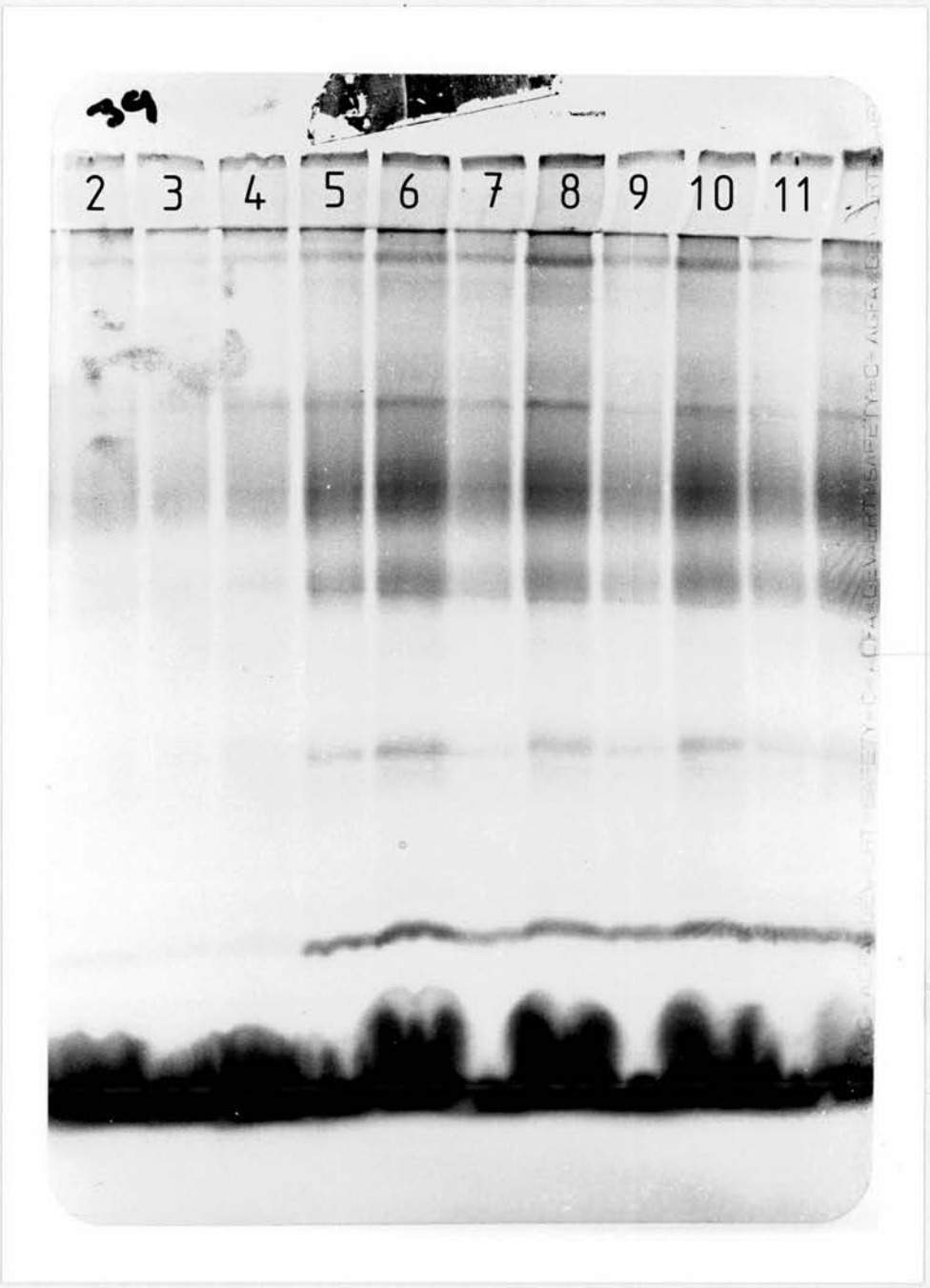


Figure 3.10. Effect of medium upon ^{14}C-DCCD incorporation into chromaffin granule membrane proteins

Autoradiograph of 10-15% exponential gradient SDS-polyacrylamide gel labelled in the media described in Table 3.3. Tracks 1 and 2 medium 1; 3 and 4 medium 2; 5 and 6 medium 3; 7 and 8 medium 4; 9 and 10 medium 5; 11 medium 6.

3.2.4. Labelling conditions

In the initial experiments on the labelling of the granule membrane with ^{14}C-DCCD it was noted that for a visible band to appear in the autoradiograph the film had to be exposed to the gel for some eight weeks. Since this was likely to determine the experimental turnover time, it would be impractical to use this method. Unless the time could be cut down, it would have been impossible to generate enough results to continue the project. One way of overcoming this would have been by the use of fluorography with PPO as a scintillator (Laskey & Mills, 1975). However the preparation of gels for PPO is a tedious process, requiring the use of dimethylsulphoxide to completely remove the water from the gel, and it also meant that it would be impossible to coomassie-stain the gels prior to or after autoradiography. At the time of these experiments the technique of fluorography with sodium salicylate (Chamberlain, 1979) had not been reported.

An attempt was therefore made to optimise the conditions for labelling of the membranes with ^{14}C-DCCD. Labelling conditions were varied as described in Table 3.3.; in this experiment the labelling was performed with variation of pH (use of Mes pH 5.5 instead of Hepes), ATP concentration (final 10 mM), and free divalent cation concentration (EDTA, Mg^{2+}). The membranes were added to give a final concentration of 1 mg ml^{-1} protein and 20 µM DCCD was added after five minutes pre-incubation of the membranes at $0^{o}C$. The membranes were then left at $0^{o}C$ for sixteen hours before SDS gel electrophoresis on a 10-15% (exponential) gradient SDS-polyacrylamide gel. After staining, the gel was dried down and subjected to autoradiography for six weeks. The results are presented in Figure 3.10. From this autoradiograph several things can be noted. First, decreasing the pH of the medium to 5.3 column 2 had no visible effect on incorporation of radioactivity. Second,

the other media which included ATP either in a form hydrolysable by the ATPase (as ATP Mg^{2+}) or as the free nucleotide in presence of EDTA, considerably enhanced the incorporation of label, not only into the low molecular weight polypeptide, but also elsewhere, several other proteins also being labelled by ^{14}C-DCCD. The result suggests that the effect of ATP is not simply an alteration in the structure of the ATPase, leading to exposure of the DCCD binding site but an overall structural effect due to charge dependent binding of ATP to the membranes. How this exposes the amino acid side chains which bind carbodiimides is unclear but the effect could be due to positive-negative interactions being broken by the phosphate anions leading to exposure of the acidic residues. Hydrophilic carbodiimides have been used as a method of assessing exposed acidic side chains in globular proteins (Hoare and Koshland, 1967) whilst denaturing the proteins causes reaction of all available glutamate/ aspartate residues as well as the c-termini.

3.2.5. Which proteins are labelled by ^{14}C-DCCD?

From Figure 3.10 it is apparent that unbound ^{14}C-DCCD and dicyclohexylurea comigrated with the lipid boundary. This lightly staining band has been shown to be lipid by use of the lipid staining dye Sudan Red IV. Radiolabel is clearly associated with the low molecular weight protein, band 61 of Abbs and Phillips (1980), termed the DCCD-reactive protein. Just above this band some contaminating mitochondrial DCCD-reactive protein is visible. Chromomembrin B (Winkler, 1976; bands 49-50 of Abbs and Phillips, 1980), which has been identified as cytochrome b_{561} (Apps et al. 1980c) is also labelled, as well as an unidentified protein, which migrates just ahead of it on the gel. The next major band which becomes labelled has been identified as a glycoprotein, bands 38-9 (Abbs and Phillips, 1980). There is also some labelling of chromogranin A

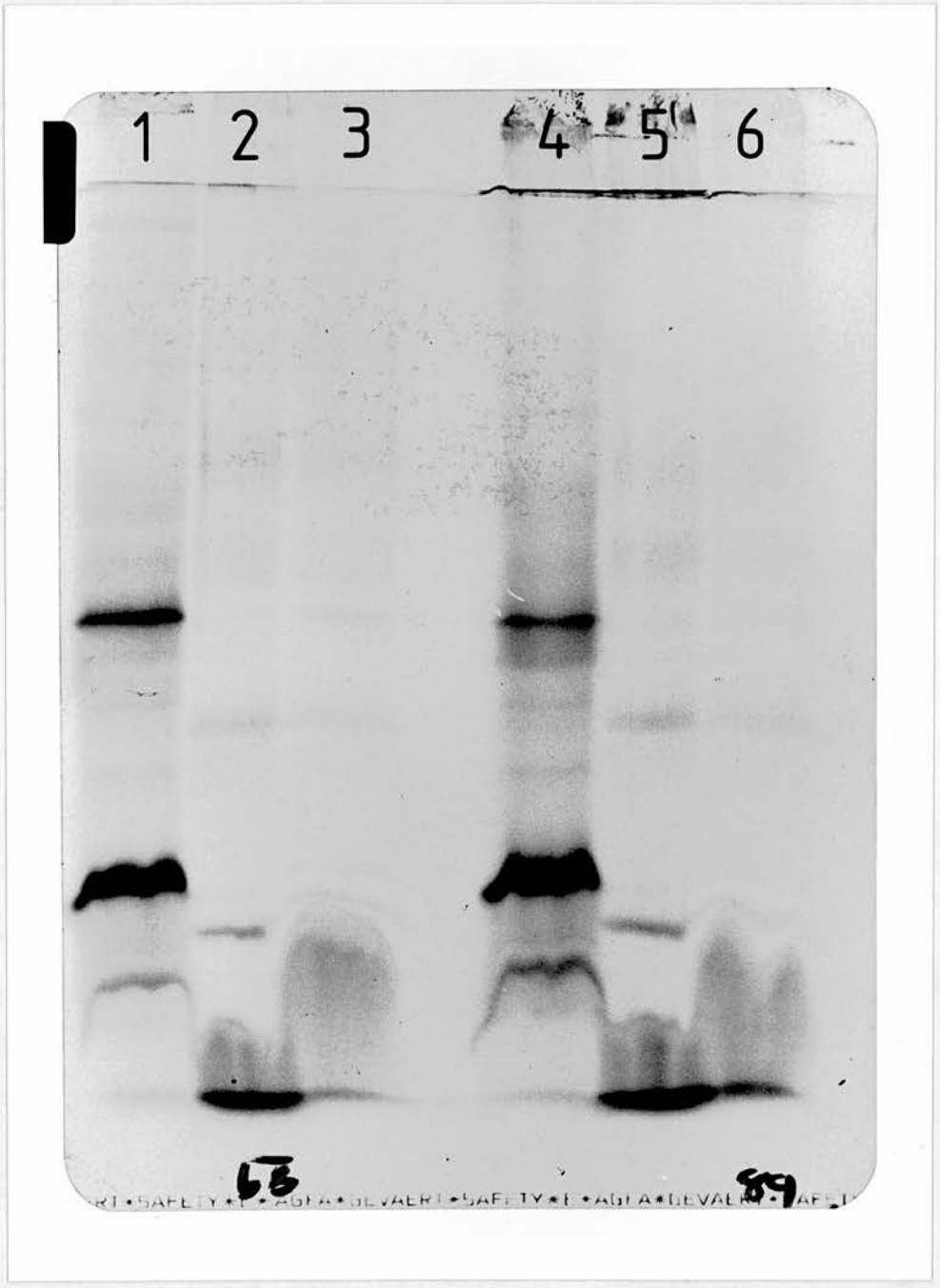


Figure 3.11. Effect of reducing conditions upon ^{14}C-DCCD pattern of chromaffin granule membranes

Autoradiograph of chromaffin granule membranes, 3 and 6: delipidated with acetone:ethanol; 2 and 5; and mitochondrial membranes; 1 and 4. All labelled with 20 µM ^{14}C-DCCD. Tracks 1-3 not reduced, 4-6 reduced with 1% v/v 2-mercaptoethanol.

(Winkler, 1976), a soluble protein from the granule matrix which may be loosely bound to the granule membrane. This is present in chromaffin granules in large amounts, but can be almost completely removed from the membranes by repeated osmotic shock. It is also a very acidic protein and thus may be prone to covalent modification by carbodiimides. Some labelled material either of very high molecular weight or more probably a mixture of aggregated proteins fails to enter the separating gel, and some even fails to enter the stacking gel.

In general the top half of the gel appears to have a darker background than the bottom half and this region of the gel probably contains poorly-resolved proteins, such as glycoproteins. More evidence concerning the existence of this protein is presented later but it appears to spread from Mr = 120,000 to Mr = 80,000. It is possible that this could be due to spontaneous reduction of dopamine β-hydroxylase or to slow entry of aggregated material into the separating gel.

An autoradiograph of reduced and non-reduced ^{14}C-DCCD labelled chromaffin granule membranes and mitochondrial membranes can be seen in Figure 3.11. It can be seen that reduction of the sample with β-mercapto-ethanol leads to an increase in the amount of material entering the separating gel. Also evident from this autoradiograph is that several mitochondrial proteins become labelled, but the largest proportion of the label can be found in the DCCD-reactive protein. However one smaller band (about 8500 mol. wt.) and one larger (30000 mol. wt.) are also labelled. The faint background labelling may well be due to nonspecific labelling of numerous proteins; for instance the α and β subunits of the ATPase are faintly labelled, as is the adenine nucleotide translocase.

Comparison of proteins electrophoresed in the presence and absence of β-mercaptoethanol shows that dopamine β-hydroxylase is labelled, as is cytochrome b_{561}. Comparison of tracks in which the chromaffin granules

were delipidated with 25 volumes of acetone:ethanol before labelling with ^{14}C-DCCD, with nondelipidated material, tracks 5 and 4 respectively, shows the presence in track 4 of a band comigrating with the Y-subunit of the ATPase. In delipidated membranes this band appears to be absent, but the label found in the cytochrome appears to be stronger. This could be due to lipid induced aggregation of the cytochrome and suggests that perhaps the cytochrome is in an oligomeric form in the granule membrane. ^{14}C-DCCD incorporated into chromogranin A, cytochrome b_{561}, and to dopamine β-hydroxylase may well be due to non-specific labelling of these proteins as they are major constituents of the granule membrane.

3.2.6. ^{14}C-DCCD-reactive site specificity.

If there is only one DCCD-reactive site per molecule of protein it should be possible by preincubation of the membranes with non radioactive DCCD, to block the binding site to ^{14}C-DCCD. If, however, binding is simply due to the attachment of a few carbodiimide molecules non-specifically to many different binding sites on any protein, the covalent binding of ^{14}C-DCCD may only be moderately affected by preincubation of the membrane with ^{12}C-DCCD. To obtain a quantitative assessment of DCCD bound to each part of the membrane, both autoradiography and gel-slicing followed by liquid scintillation spectroscopy were employed. This gave an accurate estimate of the radioactivity profiles of SDS-polyacrylamide gels of ^{12}C-DCCD labelled material.

Chromaffin granule and mitochondrial membranes were thawed after storage in Hepes/DDT/EDTA buffer at -15°C and -70°C respectively. After washing the membranes twice by ultracentrifugation and resuspension they were finally suspended at 1 mg protein ml^{-1}, 10 mM ATP. After five minutes at 4°C the 1 ml aliquots were split into two; 50 µM ^{12}C-DCCD was added to one sample from a 5 mM stock solution in ethanol, whereas ethanol was added to the control and incubated for 120 mins at 4°C. Then 5 µM ^{14}C-DCCD

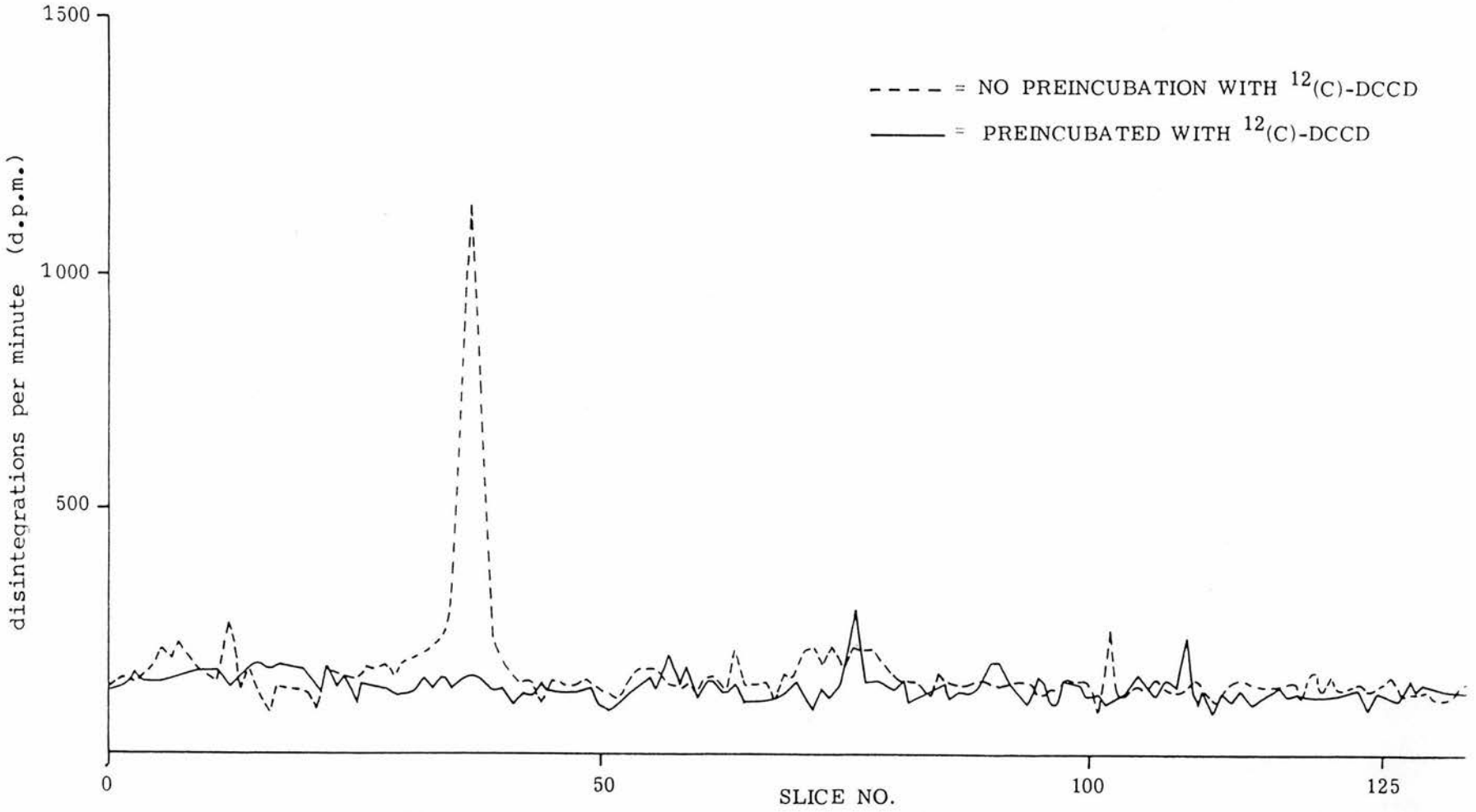


Figure 3.12. (a) Preincubation of chromaffin granule and mitochondrial membranes with ^{12}C-DCCD before addition of ^{14}C-DCCD

(a) Pattern of counts incorporated against slice number for mitochondrial membranes incubated with ^{14}C-DCCD (---) and preincubated with ^{12}C-DCCD (——) as described in the text electrophoresed on 10-15% polyacrylamide gels.

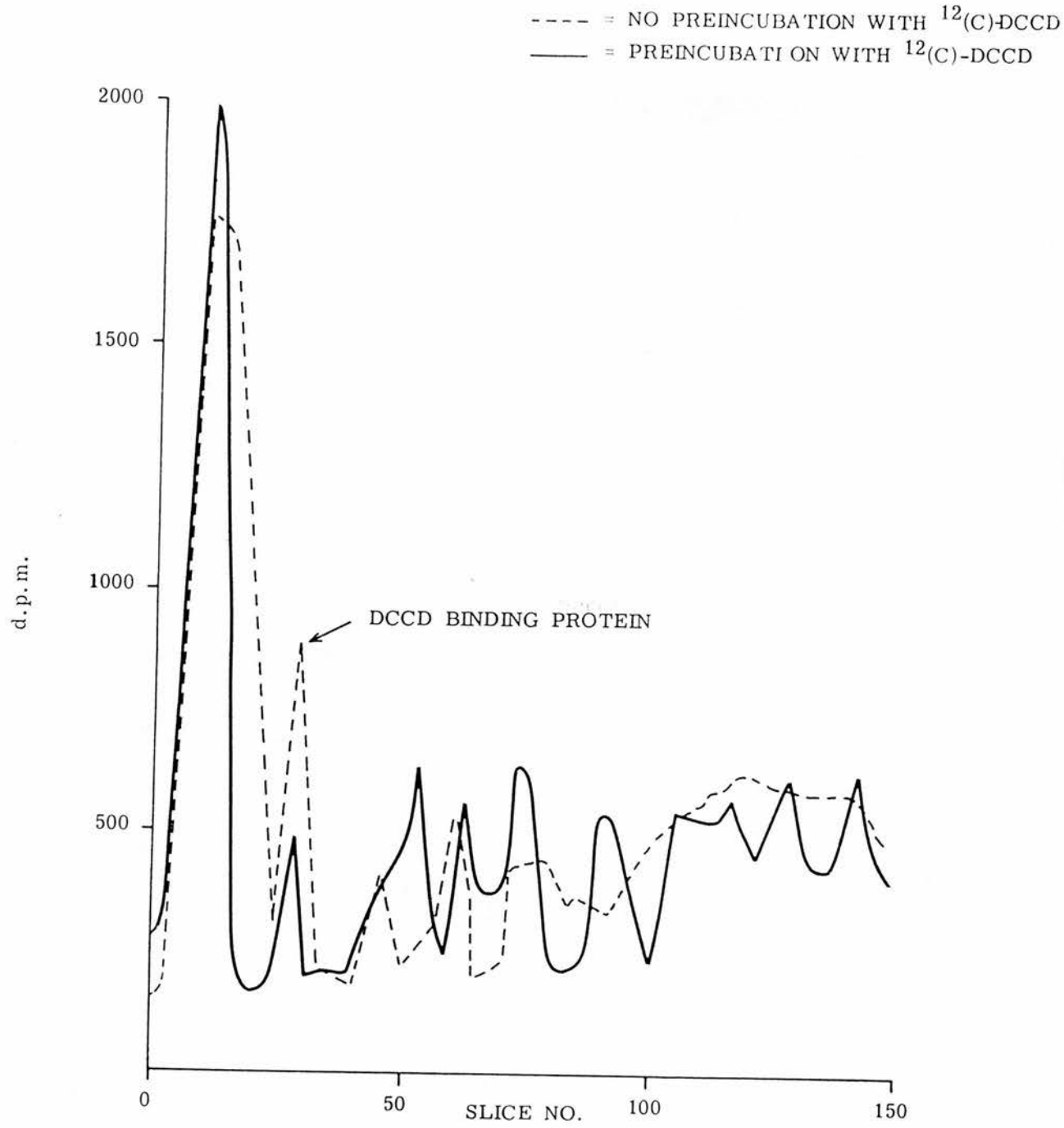
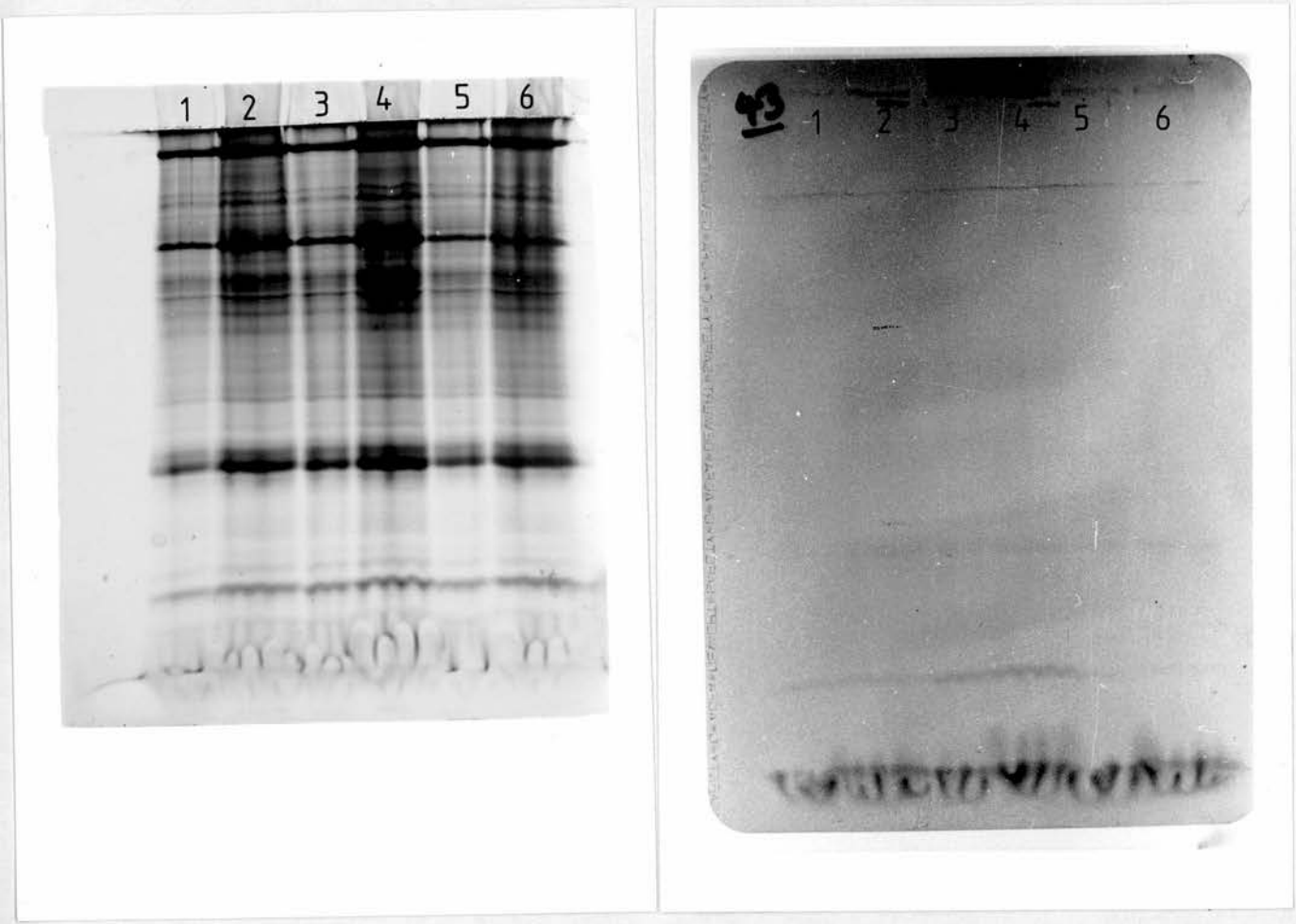


Figure 3.12. (b) Preincubation of chromaffin granule and mitochondrial membranes with ^{12}C-DCCD before addition of ^{14}C-DCCD

(b) As in (a) except that chromaffin granules were used instead of mitochondrial membranes.

was added from a 2 mM stock solution to both solutions, and the mixture incubated for 16 h at 4°C. The membranes were washed in Hepes buffer by ultracentrifugation to remove any DCCD in the aqueous phase and resuspended at 1 mg protein ml^{-1}. The membranes were solubilized in SDS sample buffer and electrophoresed on 10-15% acrylamide gels. After fixing, staining, and destaining one track was excised, scanned at 540 nm to record the coomassie blue staining pattern, and cut into 1 mM slices. The slices were then solubilized and scintillant added as described in Chapter 2. Similar tracks were then dried down and autoradiographed in order to check that counts obtained from the slicing (which had quite variable background) corresponded with the pattern obtained on the X-ray film.

The results of the slicing experiment can be seen in Figure 3.12 (a) and (b). In the mitochondrial gel track there was complete obliteration of the label incorporated into the protein on preincubation with ^{12}C-DCCD. In particular, at the concentration of ^{14}C-DCCD used only the low molecular weight DCCD-reactive polypeptide incorporated any radioactivity, and this was completely removed by preincubation with ^{12}C-DCCD. In chromaffin granules the situation appears to be different. The labelling of material at the dyefront of the gel (low molecular weight) appears to be quite unchanged by preincubation with ^{12}C-DCCD. However in the low molecular weight polypeptide (indicated by the arrow) there is quite a marked reduction in the amount of label incorporated, although there still appears to be some label incorporated after preincubation with ^{12}C-DCCD. In fact there is a very small reduction in overall incorporation in most of the proteins but a large reduction in the protein of interest, which suggests that the situation is similar to that in the mitochondria, with one class of DCCD-binding sites of high reactivity. The incomplete removal of label by preincubation with ^{12}C-DCCD could be either because the reaction is much slower in the chromaffin granule membrane protein or because there is



(a) (b)

Figure 3.13. Preincubation of membranes with ^{12}C-DCCD

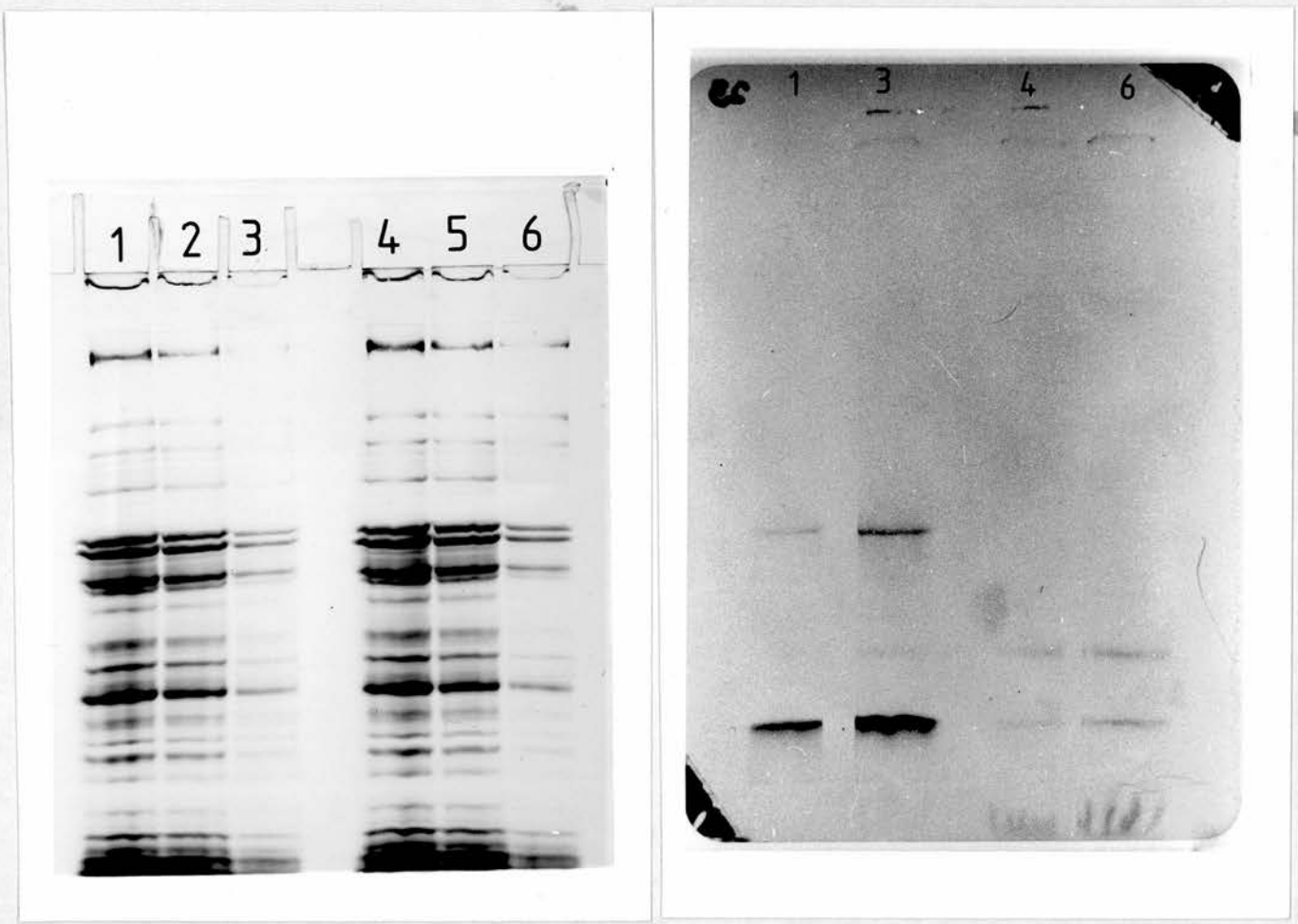
(a) 10-15% exponential gradient SDS polyacrylamide gel of ^{14}C-DCCD inhibited chromaffin granule membranes.

Tracks: 1 and 2, 2 hours preincubation with ^{12}C-DCCD;

3 and 4, no preincubation with ^{12}C-DCCD;

5 and 6, 6 hours preincubation with ^{12}C-DCCD.

(b) Autoradiograph of (a)



(c) (d)

Figure 3.13 (contd) Preincubation of membranes with ^{12}C-DCCD

(c) Mitochondrial membranes inhibited with ^{14}C-DCCD (left) but preincubated with ^{12}C-DCCD (right) electrophoresed on 10-15% exponential SDS polyacrylamide gels.

(d) Autoradiograph of (c).

some nonspecific labelling of this protein.

The specificity was explored further by preincubating the membranes for different times with ^{12}C-DCCD, to investigate the effect on binding of ^{14}C-DCCD. The experiment was essentially as described earlier except that the preincubated sample was split into two equal portions before addition of ^{14}C-DCCD, which was added after two hours preincubation and after 6 hours preincubation to the other half. SDS gel electrophoresis of the labelled material was followed by autoradiography for four weeks. The stained gel and its autoradiograph are shown in Figure 3.13 (a) and (b). Figure 3.13 (c) and (d) presents the gel and X-ray film of the initial experiment on preincubation with mitochondrial membranes. The autoradiograph shows that there is a decrease in the amount of label incorporated into the protein even after two hours preincubation and also that this decrease is greater after six hours preincubation for the chromaffin granule membrane. The idea that the DCCD-binding reaction in chromaffin granules is somewhat slower than that for mitochondria is supported by this result. It appears that at 4^{o}C the reaction needs at least twelve hours for completion, as even after six hours preincubation there is still some incorporation of ^{14}C-DCCD.

It has been reported here, with results confirmed elsewhere (L.M. Wakefield, personal communication) that ATP increases the overall amount of label incorporated from ^{14}C-DCCD into the protein, and it was of interest to see whether analogues of ATP and if so which analogues would have a similar effect on the protein-DCCD reaction. Possibly binding of ATP to the active site of the ATPase alters the overall structure of this protein, either exposing one binding site to a larger extent or exposing the same binding sites on more subunits of the same protein. The latter hypothesis follows from the observation in mitochondria and chloroplasts at low concentrations of DCCD only one of three or six

protein subunits need be covalently modified in order to achieve complete inhibition of the ATPase. This may be due to an asymmetric arrangement of the subunits to form a proton conducting oligomeric pore (Sigrist-Nelson & Azzi, 1979).

In an initial set of experiments chromaffin granule membranes were suspended at 1 mg protein ml^{-1} in the presence of 10 mM of the following ATP analogues:- GTP, CTP, UTP, ADP, PP_i, ADPNP and ATPγS. After five minutes at room temperature ^{14}C-DCCD, final concentration 20 μM, was added and the media incubated overnight at 4°C. The membranes were then washed by ultracentrifugation and resuspension at 1 mg protein ml^{-1}, dissolved in SDS sample and electrophoresed on 10-15% exponential SDS polyacrylamide gels. After fixing, staining, destaining and drying, the gel was autoradiographed for four weeks. All of the analogues were found to enhance the binding of DCCD to the protein, with the exception of ATPγS. ATPγS appears to inhibit the ATPase by binding to the active site suggesting perhaps that for the increased reaction of DCCD, binding of the β or γ phosphates of ATP to the active site of the membrane bound ATPase is important. It is odd however to note that a similar diminution of DCCD reaction was not seen using ADPNP. Possibly the thiophosphate reacts with the carbodiimide, although this possibility was not investigated further.

3.2.7. Inhibition Experiments

The experiments described above show that a protein of low molecular weight reacts with DCCD. Although it is known that labelling by DCCD is enhanced by ATP, no direct evidence has been presented to show that the protein which reacts with DCCD is part of the ATPase complex. If the reaction in the presence of ATP leads to increased inhibition of the ATPase compared to reaction in the absence of ATP, then this would provide at least indirect evidence that the binding protein is part of

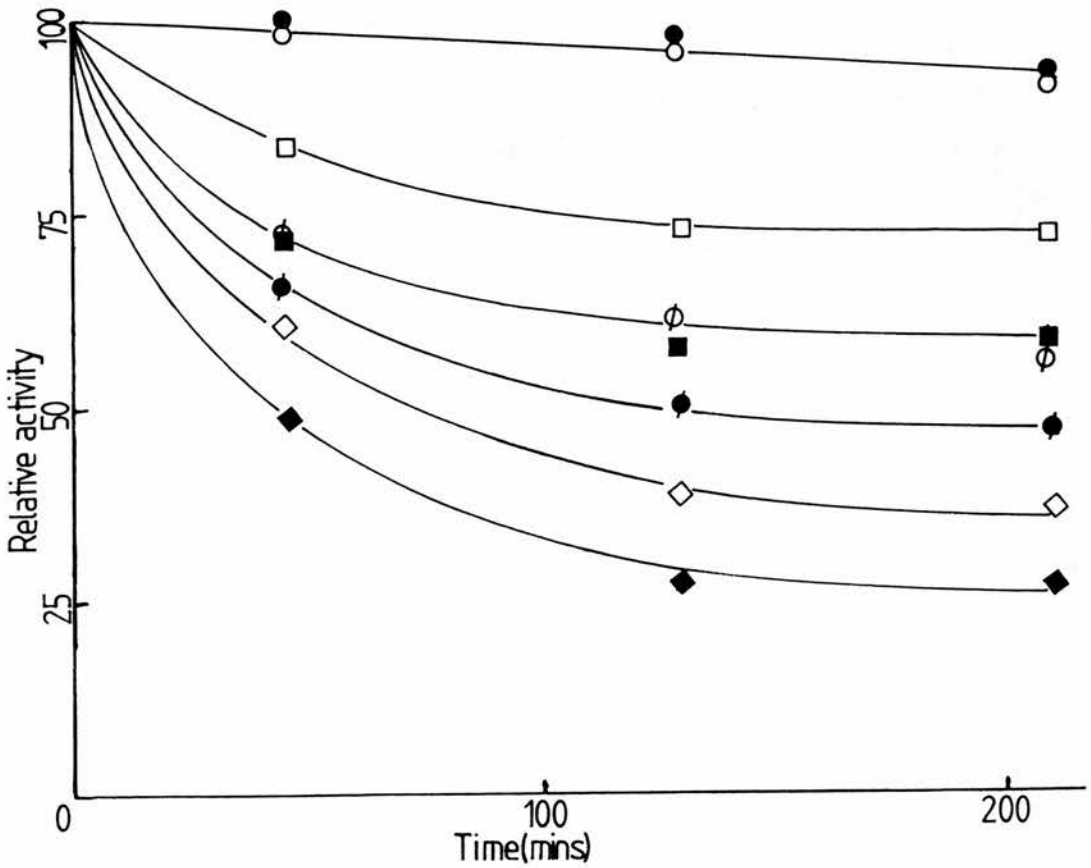


Figure 3.14. Effect of ATP on DCCD mediated inhibition of chromaffin granule ATPase activity

Time course of ATPase activity in the presence (solid symbols) and absence (open symbols) of 10 mM ATP incubated at the following DCCD concentrations 20 nmol mg protein (□, ■); 50 nmol mg protein (⌀, ⌀), 100 nmol mg protein (◇, ◆). Controls without ATP are indicated by open and solid circles.

the ATPase complex. Published material has not examined the question of covalent linkage of DCCD and all the inhibition experiments have been by measuring inhibition either of proton translocation activity or of ATPase activity (Apps et al. 1980).

Chromaffin granule membranes stored in Hepes/DTT/EDTA buffer were suspended at 1 mg protein ml^{-1}, after washing by ultracentrifugation, with or without 10 mM ATP. A DCCD stock solution was prepared freshly on the day of use by dissolving the solid in ethanol such that the highest DCCD concentration used in the experiment was a 1:100 dilution of the stock. Ethanol was also added to the controls. From the highest concentration of DCCD serial dilutions were made such that a 1:100 dilution of each would give the required concentration of DCCD (20, 50 and 100 nmoles DCCD mg $protein^{-1}$). A sample of the controls with and without ATP were taken and the ATPase activities measured at zero time, using the coupled assay described in Chapter 2. At various time intervals all the samples were assayed for ATPase activity. Control activity at zero time was 423 nmol min^{-1} mg $protein^{-1}$. The results of this experiment are presented in Figure 3.14. Even at 100 nmoles DCCD per mg protein there was incomplete inhibition of ATPase activity by DCCD. This could be due to uncoupling of the enzyme from the proton channel by partial dislodging of the F_1 like portion, which becomes DCCD insensitive, or due to the presence of a DCCD-insensitive ATPase of a completely different type in the membrane. It also appears from the control experiments that ATPase activity is lost less rapidly in the presence of ATP than in its absence. In other words, the substrate stabilises the enzyme in solution. However the most important observation is that inactivation of ATPase activity by DCCD is greatly enhanced in the presence of ATP; at low concentrations of DCCD, inhibition is increased by up to 100% on adding ATP. The slightly lessened effect of ATP at higher concentrations of

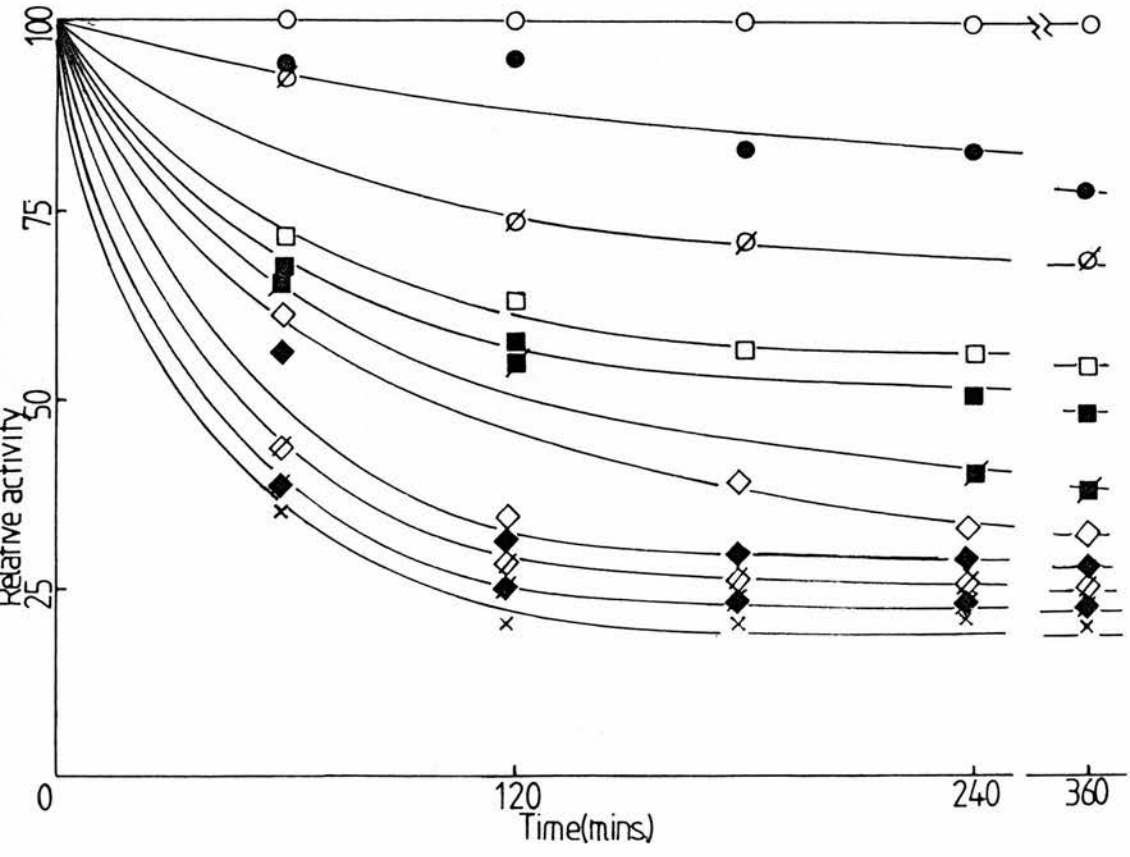


Figure 3.15. Inhibition by DCCD of chromaffin granule ATPase activity at 4°C.

Time course of ATPase activity at different concentrations (nmol mg protein^{-1}) DCCD.

o = 0 nmol; • = 5 nmol; ⌀ = 10 nmol; ◖ = 15 nmol; □ = 20 nmol; ■ = 30 nmol; ⧄ = 37.5 nmol; ◪ = 40 nmol; ◇ = 50 nmol; ◆ = 100 nmol; ⊘ = 150 nmol; ◆ = 200 nmol; x = 400 nmol DCCD:mg protein.

Starting activity was 385 nmol.min.$^{-1}$.mg protein^{-1}.

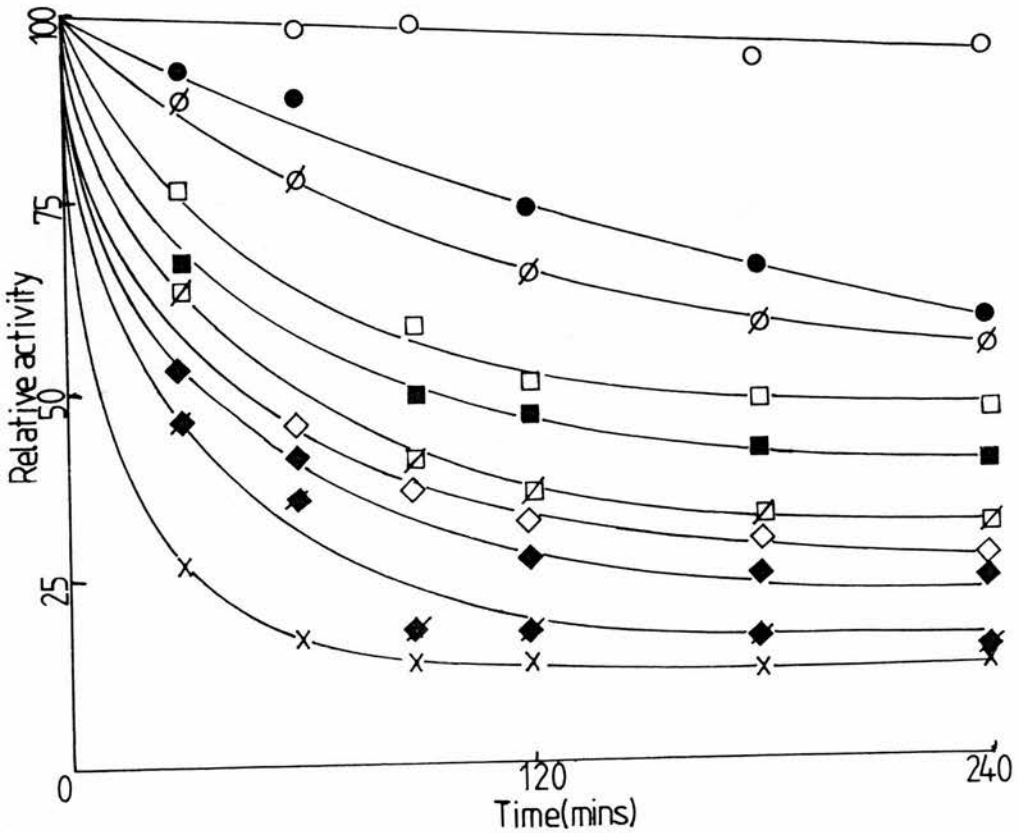


Figure 3.16. Inhibition by DCCD of chromaffin granule ATPase activity at 25°C.

Time course of ATPase activity at different DCCD concentrations. Explanation of symbols are given in Figure 3.15. Starting activity 360 nmol.min.$^{-1}$.mg^{-1}.

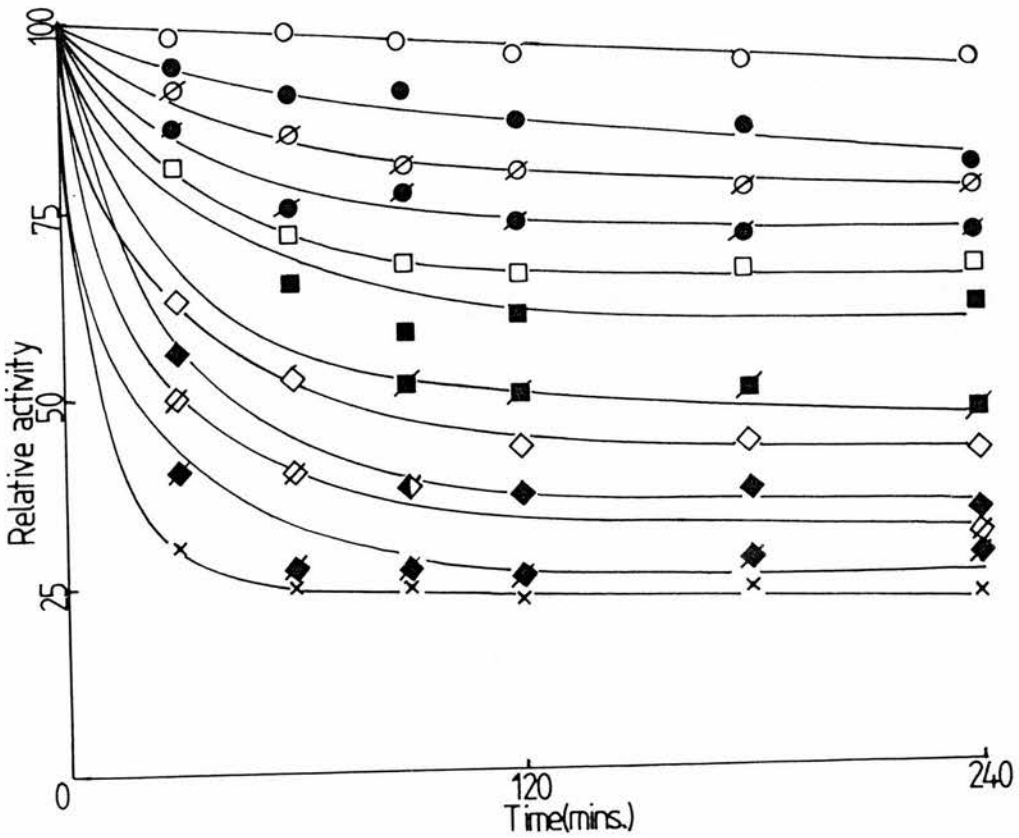


Figure 3.17. Inhibition of DCCD of chromaffin granule ATPase activity at 30°C.

Time course of ATPase activity of various DCCD concentrations at 30°C. Explanation of symbols are given in Figure 3.15. Starting activity was 423 nmol.min.$^{-1}$.mg^{-1}.

DCCD be due to nonspecific binding of DCCD to other sites on the ATPase complex, or much less specific inhibition of a second ATPase complex.

This experiment was carried out at 25°C and it was of interest to know if the extent of labelling and inhibition of the ATPase by DCCD was affected by the temperature of the incubation medium. Membrane structures appear to be quite largely affected be changes in temperature between 20°C and 30°C and also by the amount of cholesterol in the membranes, which has been shown by ^{13}C-NMR techniques (Chapman, 1975). Partitioning of DCCD into the membranes may be markedly affected by lowering the temperature, leading to hydrolysis of the carbodiimide before it partitions into the membrane; on the other hand hydration is probably accelerated at higher temperature.

Chromaffin granule membranes stored in the Hepes/DTT/EDTA buffer were washed by ultracentrifugation with the Hepes buffer, and resuspended at 1 mg protein ml^{-1}. DCCD freshly prepared and diluted as described previously was added to 1 ml aliquots giving a 1% ethanol concentration. DCCD concentrations of 0, 5, 10, 15, 20, 30, 40, 50, 100, 150, 200 and 400 nmoles DCCD were added. A sample of control membranes was taken and its ATPase activity at zero time measured. Thereafter at selected time intervals ATPase activities of all the different media were measured by the coupled assay until the reaction reached completion. Temperatures were kept constant by the reaction being carried out in 1.5 ml "microfuge" tubes in a heating block for the two higher temperatures and in glass tubes in an ice bucket for the 0°C experiment. The results are presented in Figures 3.15-17 for experiments 0°C, 25°C and 30°C. The data is also presented as concentration versus final percentage inhibition in Figure 3.18. From Figures 3.15-17 one point of interest is noticeable. Although there appears to be no difference in the final percentage inhibition at any single DCCD concentration, the reaction appears much slower at 4°C than at

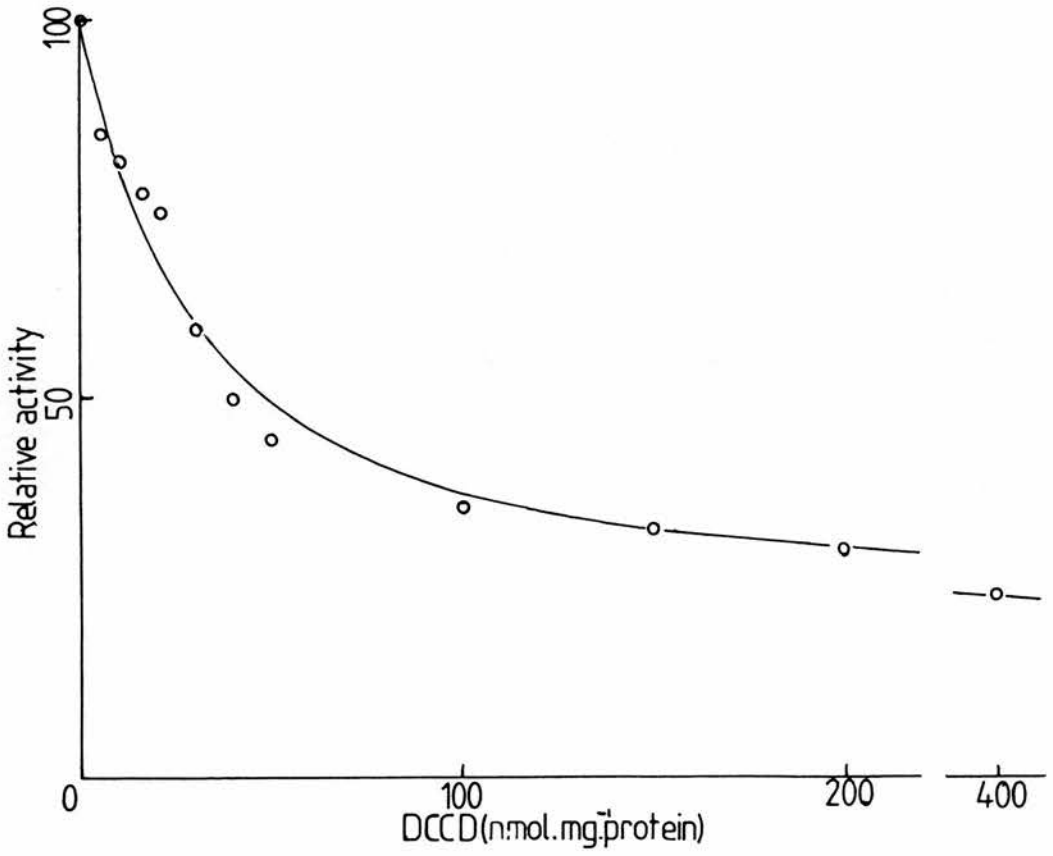


Figure 3.18. Effect of DCCD on ATPase activity of chromaffin granule membranes

Plot of concentration of DCCD against relative activity of chromaffin granule ATPase after incubation for 240 min at 25°C. Starting activity was 423 nmol.min^{-1}.mg^{-1}. Control after 240 min was 377 nmol.min^{-1}.mg^{-1}.

the higher temperatures. This result is similar to that observed in E.coli (Fillingame, 1975) or ox heart mitochondria (Beechey, 1972) in that labelling of the DCCD-reactive protein at 4°C needed longer incubation to achieve the same overall incorporation of DCCD than did labelling at a higher temperature. Figure 3.18 presents the experiment as percentage ATPase inhibited versus concentration of DCCD in the medium. The curve appears to be biphasic; at low concentrations of DCCD small increments in the DCCD concentration give large increases in the percentage of the ATPase inhibited, whilst at higher concentrations of DCCD the amount of increased inhibition of ATPase activity caused by large increments in the DCCD concentration was negligible. Two explanations offer themselves: possibly there are two distinctly different ATPases, only one of which is DCCD-sensitive, whilst the other is largely insensitive to DCCD. It would appear from experiments carried out on ghosts that, if there were two ATPases, the second ATPase must also translocate protons as part of its function as incomplete inhibition of proton translocation and ATPase by DCCD was observed (Apps et al. 1980). Alternatively, there may be only one ATPase, which has become partially detached from the proton conducting channel so that whilst ATPase activity resides in the membrane it now becomes insensitive to DCCD. Thus the small amount of inhibition seen at higher concentrations may be caused by DCCD binding to the β-subunit of the ATPase (Pougeois et al. 1980). Uncoupling of the F_1 from the ATPase could be due to the several high speed ultracentrifugation steps that the membranes undergo during routine preparation. Also experiments on ghosts show a different concentration/ inhibition profile for experiments with DCCD.

3.3. Summary

1. Chromaffin granule membranes, as routinely prepared have been shown to be contaminated less than 2.5% by mitochondrial membranes.

2. The presence of cytochrome oxidase as a component of chromaffin granule, which has been reported by other workers, has been shown to be an artefact due to mitochondrial contamination.

3. Chromaffin granules have been shown to contain a low molecular weight polypeptide which reacts with DCCD and is not a contaminant from mitochondrial membranes.

4. Molecular weight determinations based upon SDS gel electrophoresis give a molecular weight of about 14000 for the chromaffin granule DCCD-reactive protein and 15000 for the mitochondrial DCCD-reactive protein.

5. The labelling of the low molecular weight polypeptide with ^{14}C-DCCD is greatly enhanced by the presence of ATP in the incubation medium. Experiments with ATP-analogues suggest that the β phosphate of ATP may be essential for enhancement of labelling.

6. The ATPase activity of chromaffin granule membranes is more extensively and more rapidly inactivated by DCCD when ATP is present in the incubation medium. This, together with the enhanced labelling suggests that the low molecular weight polypeptide is part of the ATPase.

7. Increase in the temperature of incubation does not result in increased inhibition of the ATPase activity by DCCD but does alter the reaction rate. The results suggest either the presence of two different ATPases in the chromaffin granule membrane, or that the ATPase can be partially uncoupled from the site of DCCD reaction.

Chapter 4

Detergent solubility and chromatography of the chromaffin granule Mg^{2+}-dependent ATPase

4.1. Introduction

Investigations to find a suitable detergent to solubilise the ATPase activity of chromaffin granules is important. With this first step completed, purification of an ATPase complex, or non-denaturing two dimensional gel electrophoretic work with suitable detergents could be undertaken. Also demonstration of comigration of the DCCD-reactive protein and ATPase activity would give more convincing evidence for the presence of the DCCD reactive protein in an ATPase complex from chromaffin granule membrane. This chapter therefore first covers work on solubilisation of ATPase activity from chromaffin granule membranes, and gel chromatography of the solubilised ATPase activity.

Several workers have attempted to solubilise the ATPase activity of chromaffin granule membranes using detergents. Trifaro and Warner (1972) first described the use of sodium dodecylsulphate (SDS), Triton X-100 and Lubrol PX in solubilisation of the ATPase. They found that the best detergent was Lubrol PX, used in a 15:1 ratio of detergent: protein. This solubilised approximately 57% of the membrane protein, with an increase in specific activity of the ATPase of about twofold since 92% of the ATPase was solubilised with no significant change in the total activity. However their results show considerable inconsistency: if the activity was constant, solubilisation of 92% of the ATPase activity and 57% of the protein increases the specific activity by 56%. It is also noteworthy that the ATPase activity of the membranes before solubilisation was only 55 µmoles ATP hydrolysed $hour^{-1}$ or 16 nmoles min^{-1} mg $protein^{-1}$ at $30^{o}C$. Using a similar assay Apps and Reid (1977) found activities of 170 nmoles min^{-1} mg $protein^{-1}$, which suggests that the membranes used in the experiments of Trifaro and Warner may have had most of the ATPase inactivated, possibly a result of freezing and thawing the membranes before assay, or preparation procedure.

DETERGENT	% ATPase ACTIVITY SOLUBILISED			
	(a)	(b)	(c)	(d)
Cholate	-	69 (1.5)	49 (1)	-
Deoxycholate	90	-	36 (0.48)	-
NP40	-	-	45 (1)	
NP42	-	-	-	117 (2)
TTX 100	1.216 (0.02)	-	32 (2)	-
Lubrol PX	103 (2)		92 (1)	-
SDS	2.05 (0.5)	-	-	-

Table 4.1. Solubilisation of the chromaffin granule ATPase activity by detergent treatment. The values are expressed as activity solubilised over total ATPase activity times 100. The figures in parenthesis are % wt./wt. detergent used in the experiment.
(a) Trifaro and Warner (1972), (b) Buckland et al. (1979), (c) Giraudet et al., (1980) and (d) Apps and Reid (1977).

Buckland et al. (1979) solubilised the ATPase using 1.5% sodium cholate with 2.3 mg protein ml^{-1} of chromaffin granule membranes. The cholate soluble fraction contained 94% of the protein and approximately 60% of the ATPase activity. Apps and Reid (1977) used Nonidet P42 to solubilise the ATPase activity and found that 100% of the activity was soluble in a 2% solution of detergent, with an overall increase in specific activity of 17%. More recently Giraudet et al. (1980) used several detergents to solubilise ATPase activity. The results of all the published work are presented in Table 4.1.

Detergents have also been used for the study of the solubilisation of other membrane proteins of the chromaffin granule. Probably the best documented is dopamine β-hydroxylase, which exists both in a soluble and membrane bound form (Bjerrum et al., 1979). The membrane bound form has been successfully solubilised using low concentrations of Triton X-100 (Miras-Portugal et al., Ljones et al., 1976) or by use of lysolecithin (Aunis et al., 1977). Cytochrome b_{561} has been completely solubilised from the membranes with a mixture of two detergents: octaethyleneglycoldodecylether ($C_{12}E_8$) and cholate (Apps et al. 1980) or partially solubilised from the membranes with Triton X-100 (Silsand and Flatmark, 1974). The latter authors used a pretreatment with a less powerful detergent, Tween 20, to remove extrinsic proteins before solubilising with Triton X-100, suggesting that the cytochrome is firmly attached to the membrane. With this background it was decided to screen those detergents that were readily available, to see whether the results obtained by other workers were repeatable with membranes purified by the method described in Chapter 2.

4.2. Results

4.2.1. The assay procedure. Initially it was intended only to screen

for ATPase activity and for this the method of ^{32}-phosphate release from γ-^{32}P-ATP was deemed most suitable, compared to the colorimetric method of measuring inorganic phosphate release (Ames et al., 1975), in which formation of the phosphomolybdate complex might be severely affected by the presence of small amounts of detergent. In contrast this assay would be less subject to interference by detergents, as no complex formation is necessary, just a method of separating γ-^{32}P-ATP from free ^{32}-phosphate. However eventually a coupled assay, coupling ADP production to NADH oxidation through pyruvate kinase and lactate dehydrogenase was adopted. There are a number of advantages in using this assay in a system where the residual NADH oxidoreductase activity is very low, as it is in the chromaffin granule membrane. The method of assay of determination of $^{32}PO_4^=$ released from γ-^{32}P-ATP is a tedious procedure involving considerable time. In contrast, the coupled assay was quick, and results were obtainable almost instantly, as it is a direct spectrophotometric method. Perhaps the major advantage is that in the coupled assay the initial reaction rate is measured, and ATP is regenerated to maintain a constant substrate concentration of 1 mM; whereas in the assay for phosphate release, ATP is progressively hydrolysed to ADP, which is likely to be a competitive inhibitor of the ATPase as it is of the mitochondrial ATPase (Minkov et al., 1980). A full description of the precise assay procedures can be found in Chapter 2.

4.2.2. Detergent solubility of ATPase activity (1). Initial screening was of five available detergents using the ^{32}phosphate release assay. Chromaffin granule membranes, stored in Hepes/DTT/EDTA buffer were thawed and washed twice by ultracentrifugation to remove DTT and EDTA. No attempt was made to assess solubilisation of the cytochrome, whilst the effect of different storage conditions on the activity of the

DETERGENT	ATPase activity (nmol P_i liberated ml^{-1} min^{-1} x 10^{-2})														
	NP40			DEOXYCHOLATE			CHOLATE			TRITON X-100			TWEEN 80		
% w/v	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
0	0	0.9	7.8	0.4	1.2	11.8	0.4	0.8	11.8	1.8	1.8	11.8	0.8	0.9	9.8
0.2	0.5	2.6	19.2	0.7	2.8	16	6.6	6.7	11.3	3.6	3.8	10.8	1.9	1.2	6.3
0.5	0.6	2.5	15.7	1.6	2.8	3.8	6.8	7.1	15.4	4.4	3.6	7.2	1.7	1.8	4.0
1.0	1.5	18.7	8.9	2.1	3.0	1.8	7.9	7.9	11.5	4.8	4.4	7.2	2.5	2.4	6.0
2.0	7.8	7.4	7.16	1.6	2.0	1.1	8.8	9	8.2	5.2	5.4	2.2	0.7	0.8	10

Table 4.2. Solubilisation of chromaffin granule ATPase activity by five common detergents. Methods are as described in the text and figures are the mean of three experiments. Fractions are as follows: (a) glycerol suspended supernatants (b) water suspended supernatants and (c) pellets, resuspended in water.

solubilised ATPase was investigated, since storage of the solubilised ATPase in an active state would be a great advantage in subsequent purification steps.

The five detergents used were Nonidet P40, cholate, deoxycholate, Tween 80 and Triton X-100, in a range of concentrations from 0.2-2%. The protein concentration was fixed at 1 mg ml^{-1} (as determined by the Bradford method). 5 mls of thawed membranes (5 mg protein/ml) were washed twice and the pellets resuspended at 2 mg protein ml^{-1} final protein concentration in 100 mM Hepes NaOH pH 7.4. The detergents were added dropwise from a 10% stock solution until the final concentration was reached, the whole procedure being carried out in ice, and the final protein concentration 1 mg ml^{-1}. The solubilised membranes were then left for 30 mins at $0^{o}C$ before being centrifuged (50000 r.p.m. Beckman Ti 50 rotor, 30 mins $4^{o}C$, g_{av} = 26,000) to pellet any insoluble material. The supernatants were removed with a pasteur pipette and split into two equal aliquots, one half being diluted with an equal volume of 10% glycerol, the other half with water. The pellets were resuspended in their initial volume of Hepes buffer, by use of a Potter-Elvehjem homogeniser. After 60 minutes at $0^{o}C$ the ATPase activity was assayed by the ^{32}phosphate release method. The results are presented in Table 4.2.

Without further calculations from Table 4.2 it is quite obvious that the effect of resuspending the solubilised ATPase activity in glycerol for the duration of the experiment had little effect on preserving or activating the ATPase. For the detergents Nonidet P40 and deoxycholate a substantial reduction in the ATPase activity on storage in glycerol was observed, whilst for the other three detergents glycerol had no observable effect. Treatment of the membranes by some of the detergents resulted in an activation of the ATPase, some had no effect and others inactivated. Tween 80 had no substantial effect on ATPase activity, Nonidet P40 increased it, Triton X-100 and cholate

DETERGENT	% ATPase activity solubilised				
% w/v	NP40	Deoxycholate	Cholate	Triton X-100	Tween 80
0	0	0	0	0	0
0.2	2.56	8.54	42.21	17.98	12.07
0.5	5.40	42.09	36.03	26.62	32.16
1.0	62.02	46.43	46.94	31.62	28.92
2.0	48.65	61.07	56.76	46.75	-1.73

Table 4.3. Solubilisation of ATPase by five common detergents at various different detergent concentrations. The results are expressed as % total ATPase activity solubilised as calculated from Table 4.2. as described in the text.

activated up to a certain detergent concentration (0.2 and 0.5% respectively), whilst deoxycholate appeared to inactivate.

The ability of individual detergents to solubilise ATPase activity is shown in Table 4.3. The results are expressed as percentage of the total ATPase activity solubilised, with the figures taken being those of the activities resuspended in water, rather than glycerol. The equation used to calculate the values in the table is:

$$\frac{\text{ATPase activity in the supernatant} \times 200}{\text{Total ATPase activity (pellet + supernatant)}}$$

The reason for the 200 is due to the dilution in resuspending half in water and a percentage conversion. Interpretation of the results rests on the assumption that there is no change in ATPase activity when pelleted membranes are resuspended in water or diluted in water from the supernatant in centrifugation of solubilised membranes. This may result in inaccuracies when the solubilised ATPase is inactivated by the detergent used, as is the case for Nonidet P40 and Tween 80. Similar results are obtained with deoxycholate and cholate, both giving about 60% of the activity solubilised whereas Triton X-100 solubilised 46% of the total ATPase activity. The overall conclusion from this initial experiment was that no detergent was effective at solubilising all of the ATPase activity, whilst maintaining the ATPase in an active form.

In order to investigate the effect of the detergent Nonidet P40 further, a simple experiment was performed in which the detergent concentration was kept at constant level whilst the protein concentration was varied, up to 1 mg ml^{-1}. ATPase activities of the supernatants and pellets from centrifugation of the solubilised membranes were then measured. The experiment was performed in a similar way to the previous experiment.

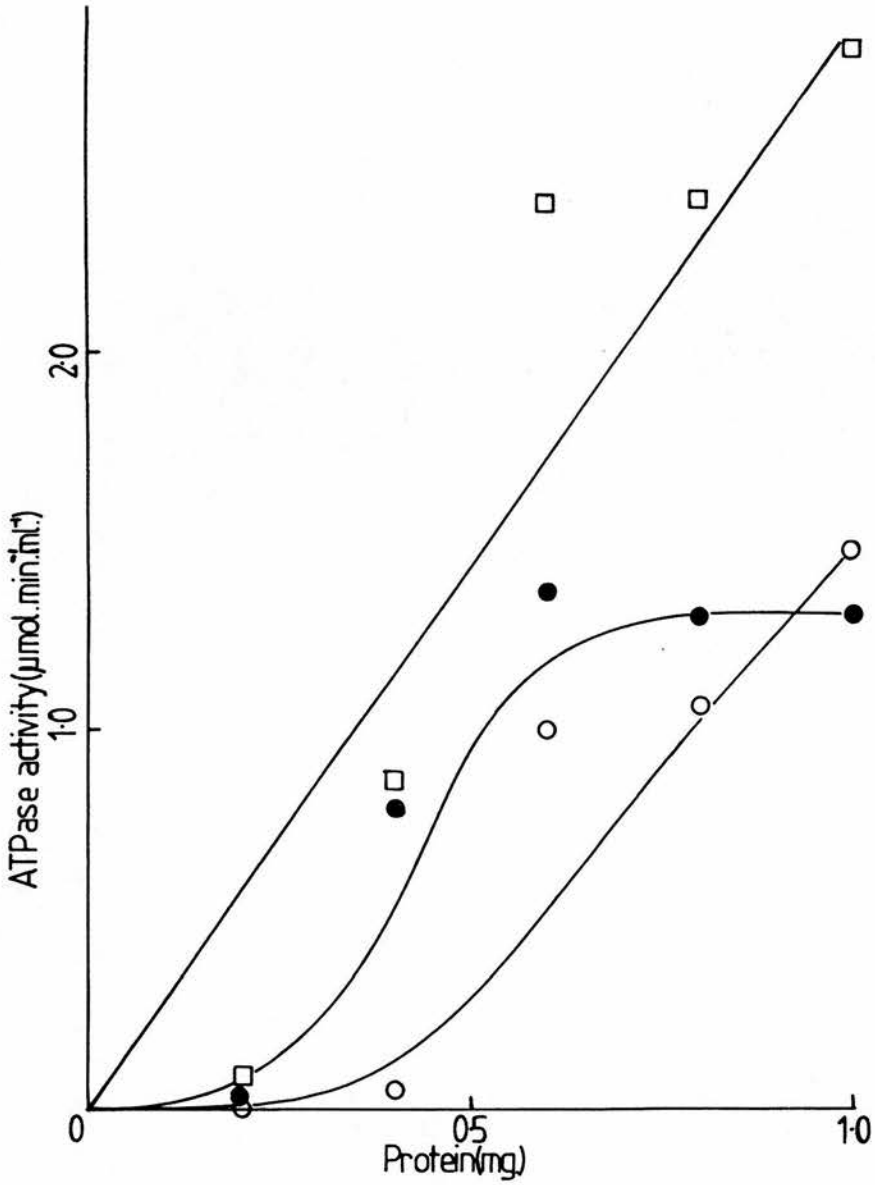


Figure 4.1. Solubilisation of ATPase activity by 2% wt./vol. NP40 at different protein concentrations.

The graph shows activity solubilised (●), activity in the pellet (o) and total activity (◘). The experiment and calculations are described in the text.

Method. Chromaffin granule membranes, stored in Hepes/DTT/EDTA buffer, -15°C, were thawed, washed by centrifugation and resuspended at 2 mg protein ml^{-1} in Hepes buffer. Aliquots were taken and diluted to give 0.2 to 1.0 mg protein ml^{-1}, and an equal volume of 4% w/v Nonidet P40 added in drops to each solution, which was left to stand for 30 mins. The solutions were then centrifuged (50000 r.p.m. Beckman Ti 50 rotor, 4°C, 30 mins, g_{av} = 26000). The superantants were removed and the pellets resuspended in one volume of Hepes buffer. ATPase activities of supernatants and pellets were measured using the ^{32}phosphate release method, and results of this experiment presented in Figure 4.1. From this experiment it is quite clear that there was no inactivation of the ATPase at any concentration. In fact the results suggest that there is an optimum concentration ratio of protein:detergent at which the maximal amount of solubilisation is achieved and above which a smaller fraction of the activity is solubilised. For Nonidet P40 this appears to be about 1:70. However it can be seen that at very low protein: detergent ratios (i.e. less than 1:100) then even Nonidet will solubilise all of the ATPase. The inhibitory effect noted in the previous experiments suggests that inhibition may be a result of solubilisation of the ATPase at too high an overall protein concentration.

Finally it is worth making a brief comparison of these results with those obtained by other workers on solubilisation of chromaffin granule ATPase activity with detergents. Since there is no published work on Tween 80, whilst this experiment contains no results with Nonidet P42 or Lubrol PX, comments are restricted to the remaining detergents. The result for Nonidet P40 agrees closely with the work of Giraudet et al. (1980), as does the figure for cholate; however the values obtained by these authors for Triton X-100 and deoxycholate disagrees with the results presented here. It appears that none of the

work is compatible with the results published by Buckland et al. (1979) or with those of Trifaro and Warner (1972). The main conclusion from this initial work is that none of the detergents used were particularly good at solubilising ATPase activity and so it was necessary to try a wider range of commercially available detergents, if solubilisation of the ATPase was to be achieved.

4.2.3. Detergent Solubility of ATPase activity (2). The primary purpose of this experiment was to screen a much wider range of detergents, and so nineteen commercially available detergents were tried. As this was a much wider range of detergents a less thorough screening procedure was used, which consisted of fixing the detergent at 2% w/v and protein at 1 mg ml^{-1}, giving a 20:1 w/w ratio of detergent to protein. The assay system used was the NADH linked coupled assay described in materials and methods. However since in this experiment detergent would be added to the assay mix, the assay was checked by adding detergents in an ADP solution to see if the assay system was affected by the detergent. It was found that the assay was seriously affected by the addition of cetyl triethyl ammonium bromide (CTAB) to the mixture, whilst the other detergents used had little or no effect on the assay. Thus results with CTAB have been limited to the assay for cytochrome b_{561}. It was assumed that assays of the cytochrome would act as a guide for selectivity of the detergent as it has been shown (Abbs and Phillips 1980, Apps et al. 1980) to be an integral membrane protein.

Solubilisation Procedure. Chromaffin granule membranes stored at $-15^{o}C$ in Hepes/DTT/EDTA buffer were thawed and washed by ultracentrifugation with Hepes buffer, and a protein estimation performed on the resuspended pellet (Bradford, 1976). The membranes were resuspended at 2 mg protein ml^{-1} in Hepes, and detergent added dropwise to give a final

Detergent 2% except when stated	ATPase nmol/min/ml		Cytochrome b_{561} ng/ml		Protein mg/ml		% solubilised			Specific activity nmol/min/mg				% Start Activity
										ATPase		cyt b_{561} µg/ml		
	SN	P	SN	P	P	SN	ATPase	cyt b_{561}	protein	SN	P	SN	P	ATPase
Tween 20	76	731	0.1	1.3	0.83	1.27	9	7	60	60	880	0.07	1.57	96
Tween 80	33	670	0.3	4.9	1.28	0.69	4.7	6	35	47	524	0.43	3.82	84
Tween 85	84	476	0.1	2.8	1.44	0.53	15	3	26	158	330	0.19	1.94	67
NP 40	282	306	2.2	3.7	0.73	1.26	48	37	63	223	456	1.74	5.00	70
NP 42	268	338	4.3	3.1	0.87	1.18	44	58	58	227	389	3.64	3.56	72
Titon X 100	38	56	2.7	1.9	0.74	1.33	40	58	63	29	77	2.03	2.86	11
Cholate	195	335	1.6	3.6	0.37	1.79	37	33	83	108	567	0.89	9.72	64
Deoxycholate	149	15	4.9	1.8	0.63	1.40	90	73	69	106	23	3.50	2.85	40
S.D.S. (0.05)	17	224	0.4	7.3	1.02	0.84	9.2	5	45	20	905	0.48	6.89	30
CTAB (0.1)	21	326	0.2	4.1	0.42	1.52	6	5	78	14	776	0.13	9.76	86
Lubrol PX	158	158	3.7	3.3	0.30	1.72	50	53	85	92	526	2.15	11	77
Lubrol LX	47	595	0.6	6.2	0.53	1.46	7	9	73	32	1122	0.41	11.70	156
β Octyl.Gluc.	56	27	7.8	1.4	0.32	1.66	67	85	84	248	578	4.69	4.37	10
$C_{12}E_8$	412	185	5.6	1.0	0.52	1.43	69	85	73	288	356	3.91	1.92	71
Digitonin (0.2)	10	268	0.1	6.3	1.10	0.92	3	2	46	11	283	0.11	5.73	33
Brij 36T	248	167	5.2	1.7	0.59	1.62	60	75	73	153	283	3.21	2.88	50
Lysol.	103	345	2.0	7.3	0.9	1.14	23	21	56	90	383	1.75	8.11	54
Oct.glu./cho	145	12	9.0	0.7	0.38	1.64	92	92	81	88	32	5.49	1.84	19

Table 4.4. Solubilisation of chromaffin granule ATPase activity and cytochrome b_{561} by a variety of different detergents.

The table shows ATPase activities, % solubilised, specific activities, and the percentage of the membrane activity. Starting activity was 836 nmol min^{-1} ml^{-1} for ATPase. Values for the cytochrome are expressed in ug based on a molecular weight of 23500 for the cytochrome (Apps, Pryde and Phillips, 1980).

concentration of 2% w/v detergent, 1 mg protein ml^{-1} and 100 mM Hepes NaOH pH 7.4. This solution was then centrifuged (50000 r.p.m., 30 mins, 4°C, Beckman Ti 50 rotor, g_{av} = 26000). The supernatant from the centrifugation was removed, and the pellet resuspended in one volume of Hepes buffer. ATPase activity and cytochrome b_{561} content were assayed by the method described in Chapter 2 (type I and type II assay respectively). Initially protein estimations were performed using the Bradford (1976) assay procedure, but experiments on the effect of detergent on the assay show its unreliability in the presence of even small amounts of detergent (see Chapter 2, type I assay). So, the results have been based on a modified Lowry assay (Hartree, 1972) which removes residual detergent and lipid, before assaying for protein in the presence of SDS. The results are presented in Table 4.4.

From this Table, which lists the percentages of the ATPase activity solubilised. it can be seen that only four detergents gave more than 70% solubilisation. They were $C_{12}E_8$, β-octylglucoside, cholate and deoxycholate. Only $C_{12}E_8$ gave a good preservation of the ATPase activity, percentage activity preserved being calculated as follows:

$$\frac{\text{Activity of supernatant + Activity of Pellet}}{\text{Activity of native membranes}} \times 100$$

In comparison with the previous experiments it should be noted that the results obtained for cholate and deoxycholate differ quite markedly, probably due to differences in the experimental protocol, but results for the other detergents matched quite well. Examining the results obtained by other workers, the value for deoxycholate agrees quite well with the results of Trifaro and Warner, but not with those of Giraudet et al., whilst the results obtained with Lubrol PX disagree with both these authors. The results obtained by Giraudet et al. for Nonidet P40

and Triton X-100 agrees closely with the results presented here. For Nonidet P42 no correlation with the results obtained by Apps and Reid (1977) could be found. It should be further noted that the precise solubilisation conditions used here differed from other workers with respect to salt and buffer concentrations and to temperature at which the solubilisation was carried out, and this may well have a considerable effect on the solubilising properties of each detergent. This will be considered later.

For the cytochrome b_{561} solubilisation there is little published material for comparison. The results obtained by Apps et al. (1980) for the detergent mixture $C_{12}E_8$ and cholate agree quite well with results obtained here using a β-octylglucoside, cholate mixture, suggesting that mixtures of ionic and nonionic detergents may serve as extremely good solubilsing agents. The result of 58% solubilisation of cytochrome with Triton X-100 agrees well with the figures published by Silsand and Flatmark (1974) of 54-60%, as do the data for Tween 20 solubilisation of the cytochrome.

4.2.4. Detergent Selectivity. One of the objectives in using such a wide variety of detergents was to find a detergent that, whilst solubilising most of the loosely bound proteins from the membrane left the integral proteins such as the ATPase and cytochrome b_{561} intact in the membrane. From Table 4.4 there are two detergents which do this and also preserve the ATPase activity: Tween 20 and Lubrol WX. Whilst SDS, CTAB, Tweens 80 and 85 also remove a large proportion of the protein without solubilising the ATPase, the activity of the ATPase is markedly reduced by the action of these detergents. If one examines a polyacrylamide gel electrophoretogram of membranes which had been prewashed (not shown) there was considerable similarity between the detergent-solubilised protein, and granule lysate. Two of these bands have been identified as

DETERGENT 2% unless stated	ACTIVITY nmol/min/ml SN	P	SN+P	% ATPase SOLUBILISED	$\frac{SN+P}{904} \times 100$
Tween 20	63	1005	1068	5.9	118
Tween 80	18	873	891	2.0	99
Tween 85	0	648	648	0	72
NP 40	558	490	1048	53	116
NP 42	324	670	994	33	110
Triton X-100	515	187	705	73	78
Cholate	448	167	615	73	68
Deoxycholate	256	69	325	79	36
S.D.S. (0.05)	51	837	888	5.7	98
CTAB (1.0)	-	-	-	-	-
Lubrol PX	628	69	697	90	77
Lubrol WX	536	332	858	62	95
$C_{12}E_8$	1125	96	1221	92	135
Digitonin	52	473	525	9.9	58
Brij 36T	1057	98	1155	92	128
Lysolecithin	618	416	1034	60	114
Saponin	32	744	776	4.1	86
OCTYL.GLU./CHO(2/0.5)	55	54	109	50	12

Table 4.5. Solubilisation of chromaffin granule ATPase activity by a variety of different detergents at 10 mM Hepes concentration

The table shows overall activity of fractions, percent of the ATPase activity solubilised and percentage of the starting activity found in the solubilised membranes and pellets. The starting activity was 904 mmol min^{-1} ml^{-1} for the ATPase.

dopamine β-hydroxylase and chromogranin A (Winkler, 1976), and analysis of the coomassie staining pattern reveals them also as major constituents of the membrane. This result fits quite well with the idea that the mild detergent treatment removes only extrinsic or loosely bound proteins, and also suggests that the protein estimations accurately measured the release of soluble protein by the detergents, rather than being an artefact caused by the detergents themselves.

4.2.5. Effect of Hepes Concentration. In their review of the action of detergents, Helenius and Simons (1975) showed that increasing solute concentration has a marked effect on the overall properties of the detergent in solution. In experiments 1 and 2 the Hepes concentration was kept quite high to give a large buffering capacity when solubilising the membranes with detergents. Initial experiments on solubilisation of chromaffin granule membranes by Tween 20 and Lubrol PX showed quite clearly that altering the Hepes concentration had a marked effect. In a further experiment solubilisation of chromaffin granule membranes with detergents at a fixed Hepes concentration of 10 mM was tried.

Method. Chromaffin granule membranes stored at -15°C in Hepes/DTT/EDTA buffer were thawed, washed by ultracentrifugation, resuspended in Hepes buffer, and protein in the resuspended pellet determined by the Bradford assay. The membranes were suspended at 2 mg protein ml^{-1} in 10 mM Hepes-NaOH pH 7.4 and detergent added dropwise to give a final concentration of 1 mg protein/ml, 2% w/v detergent, and 10 mM Hepes NaOH pH 7.4. After 5 mins at 4°C the solubilised membranes were centrifuged (30 mins, 50000 r.p.m., Beckman Ti50 rotor, 4°C g_{av} = 26000). The supernatant from the centrifugation was removed, and the pellet resuspended in one volume of Hepes buffer. ATPase assays were performed on the supernatants and resuspended pellets by the coupled assay. The results of this experiment are presented in Table 4.5.

By comparison with Table 4.4, with the exception of saponin (not used before) and CTAB (use of the larger concentration in this experiment results in inactivation) detergents could be divided into three classes. Those in which the increased salt concentration led to a greater solubilisation of the ATPase were Nonidet P42, deoxycholate, SDS, and the β-octylglucoside/cholate mixture. Those, which upon increasing the salt concentration had no effect were Tweens 20, 80 and 85, Nonidet P40 and Digitonin. For the remaining detergents increasing the salt concentration lowered the degree of solubilisation of the membrane quite markedly. Exactly whether the effect is due to increasing ionic or osmotic strength is in some doubt as Hepes is close to its pKa value at 7.4, but it must be assumed that most of the Hepes is in a zwitterionic form.

In comparison with other results the value obtained with cholate is very similar to that published by Buckland et al. (1979), whilst the results obtained by use of Nonidet P40 and Lubrol PX are similar to those found by Giraudet et al. (1980). The low solubilisation observed by Giraudet et al. for cholate may be due to the use of 0.2M KCl in their solubilisation mixture. The results for NP42 agree quite well with those of Apps and Reid (1977), bearing in mind that in their experiment the solubilised membranes were not separated from the nonsolubilised membranes by centrifugation of the detergent solubilised material. The result of SDS agrees quite well with the figures published by Trifaro and Warner, although their results may be difficult to interpret, as discussed in the introduction to this chapter. The overall impression is that the results obtained here agree quite well with those of other workers.

4.2.6. Two step solubilisation of ATPase activity. In their purification of cytochrome b_{561}, Silsand and Flatmark (1974) described a two step

DETERGENT IN SECOND STEP	TWEEN SUPERNATANT			SECOND STEP FRACTIONS					
	ATPase nmol./ml	$cyt.b_{561}$ ng/ml	Protein mg/ml	ATPase nmol/min/ml		$cyt.b_{561}$ ng/ml		Protein mg/ml	
				SN	P	SN	P	SN	P
	24	0	0.9						
$C_{12}E_8$				720	268	6.36	1.69	0.78	0.33
NP42				402	474	4.09	3.89	0.62	0.52
Deoxycholate				312	32	5.37	2.83	0.71	0.42

Table 4.6. (a). Solubilisation of chromaffin granule membranes in a two step method.

Table gives protein, cytochrome and ATPase activity, of important fractions. SN = supernatant, P = pellet. Method as described in the text. Starting ATPase activity was 660 nmol min^{-1} ml^{-1}.

SPECIFIC 'ACTIVITIES'		$C_{12}E_8$	Nonidet P42	Deoxycholate
ATPase Tween SN	)	27	27	27
ATPase second step SN	) nmol min^{-1} mg^{-1}	924	648	440
ATPase second step P	)	868	912	76
Cytochrome b_{561} Tween SN	)	0	0	0
Cytochrome b_{561} second step SN	) ng ml^{-1}	8.15	6.59	7.56
Cytochrome b_{561} second step P	)	5.12	7.48	6.73

Table 4.6. (b) Specific activity of ATPase fractions calculated from Table 4.6.(a) and cytochrome b_{561} calculated from Table 4.6.(a). Starting specific ATPase activity was 330 nmol min^{-1} mg $protein^{-1}$.

procedure for solubilisation of this protein. This involved an initial wash of the membranes in 2% Tween 20 followed by solubilisation of the cytochrome using Triton X-100. In their experiment, the effect of first solubilising the chromaffin granule membranes with mild detergent, followed by complete solubilisation with a different detergent was attempted.

Method. Chromaffin granule membranes, stored at -15°C in Hepes/DTT/EDTA buffer, were thawed and washed as previously described (experiment 4) and resuspended at 2 mg protein ml^{-1}. Tween 20 was added to give 2% w/v detergent, 1 mg protein ml^{-1}, 100 mM Hepes NaOH pH 7.4. The solution was left for 30 mins either at 25°C or 0°C before ultracentrifugation (50000 r.p.m. Beckman Ti50 rotor 30 mins 4°C g_{av} = 26000). The supernatants were removed and assayed for ATPase activity, cytochrome b_{561} (type I assay) and protein (Lowry assay) as previously described. It was found that incubation at 0°C gave the best protein/ATPase ratio in the initial solubilisation of peripheral protein and so the results are based on protein presolubilised at 0°C. The supernatant was removed and the pellets were resuspended at their initial volume (half solubilisation volume). $C_{12}E_8$, Nonidet P42 or deoxycholate were added to give a final detergent concentration of 2% w/v in 10 mM Hepes NaOH pH 7.4. The solutions were left for 30 mins at 25°C before ultracentrifugation as previously described. The supernatants were then removed and the pellets resuspended in their initial volume. Cytochrome b_{561} and protein contents were assayed, as was the ATPase activity of both the pellet and supernatants. The results are presented in Tables 4.6(a) and (b).

Of immediate note is the activation of ATPase activity by the detergents Nonidet P42 and $C_{12}E_8$, whilst deoxycholate inactivated the enzyme as previously observed. Also noteworthy is that the initial

treatment of the membranes with Tween 20 radically altered the subsequent membrane solubilisation procedure, decreasing the percentage of the ATPase activity solubilised by $C_{12}E_8$, and having the opposite effect on Nonidet P42 and deoxycholate. The increase in specific activity of the ATPase found by this method using $C_{12}E_8$, when corrected for ATPase activation, was about two fold. So this technique was partially successful, increasing the specific activity of the ATPase without giving any great degree of protein purification, as could be seen by SDS polyacrylamide gel electrophoresis of the solubilised membrane proteins (not shown). It is of interest to compare the cytochrome result with that obtained by Silsand and Flatmark (1974). They reported that some 45% of the total protein was solubilised by Tween 20 with virtually no solubilisation of cytochrome b_{561}, which agrees with the results presented here. However they only obtained 55% solubilisation of the cytochrome with Triton X-100, and $C_{12}E_8$ appeared to be an equally good if not better solubilising agent than Triton X-100. A modification of the use of $C_{12}E_8$ has been more recently used as the basis of a purification method for the cytochrome (Apps et al., 1980c) whereby initial solubilisation was achieved by use of $C_{12}E_8$ and cholate mixture.

4.3. Chromatography of Detergent Solubilised Chromaffin Granule Membranes

4.3.1. There were two main aims in the chromatography of detergent solubilised membranes. It could serve as a first stage in purification of an active F_oF_1 ATPase complex, followed by reconstitution into liposomes or characterisation of its subunit structure. Also chromatography could be used to show that the DCCD reactive protein comigrates with either ATPase activity or F_1 ATPase. Molecular exclusion chromatography on Sephacryl 300 was used as the basis of this work.

4.3.2. Molecular Weight Calibration of the Column. Sephacryl 300 was poured into a 90 x 2.2 cm column at room temperature by loading slurry at

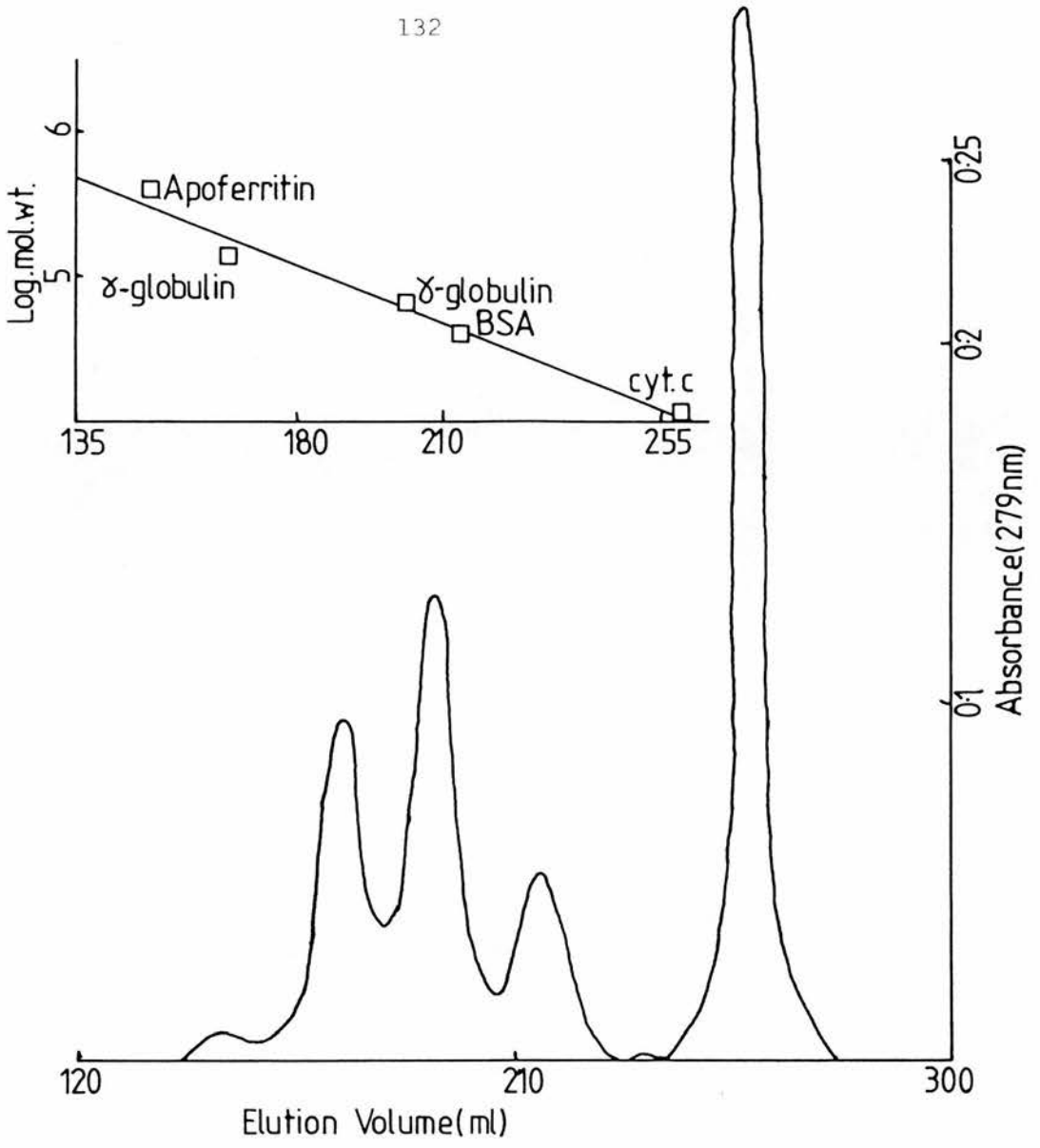


Figure 4.2. Molecular weight calibration of Sephacryl 300 column with standard proteins.

2 mg of apoferritin, γ globulin, bovine serum albumin, and cytochrome c were eluted from the column with column buffer as described in the text. The insert shows a graph of molecular weight against elution volume for the standard proteins. γ-globulin eluted from the column both as native enzyme and heavy chain dimers.

at the same time as solute was eluting from the bottom. After packing was complete the column was removed to the cold room. All experiments with the column were performed at 4°C. The column was equilibrated in 10% v/v glycerol, 0.01% w/v $C_{12}E_8$, 2 mM ATP, 0.1 mM EDTA, 0.1 mM DTT, 0.2 M KCl, 20 mM Hepes NaOH pH 7.4 ("column buffer") and 2 mg of the following proteins loaded onto the column in 2 ml; horse heart apoferritin, bovine serum albumin, rabbit Y-globulin and horse heart cytochrome c. Elution with column buffer was continued at 0.3 ml/min, until cytochrome c was seen in the eluate. The eluate was scanned for protein at 280 nm and the result of this experiment is shown in Figure 4.2(a) and (b). A plot of logarithm of molecular weight against elution volume gave a linear relationship and was used for molecular weight determinations. However it must be noted that apoferritin eluted near to the void volume, and thus calibrations of molecular weights of greater than 400000 may be unreliable with sephacryl 300.

4.3.3. Chromatography of $C_{12}E_8$ solubilised chromaffin granule membranes

In this experiment the aim was simply to visualise the protein and ATPase activity profiles of solubilised chromaffin granule membranes.

Method. The column used was as described in 4.3.2. equilibrated in column buffer. Chromaffin granule membranes, prepared freshly as described in section 2.1 were centrifuged in 10 volumes of Hepes buffer and resuspended in a minimum volume of Hepes buffer. Protein estimation was now performed and $C_{12}E_8$ added dropwise from a 10% w/v stock solution to 1 ml of the chromaffin granule membranes to give a final protein to detergent ratio of 1:5 w/w. The solubilised membranes were centrifuged (50000 r.p.m., 30 mins. Beckman Ti50 rotor, 4°C, g_{av} = 26000) to pellet insoluble material. The supernatant was removed and loaded onto the column, a small sample being kept at 4°C for subsequent ATPase assay. The solubilised material was eluted with column buffer at 4°C into 3 ml

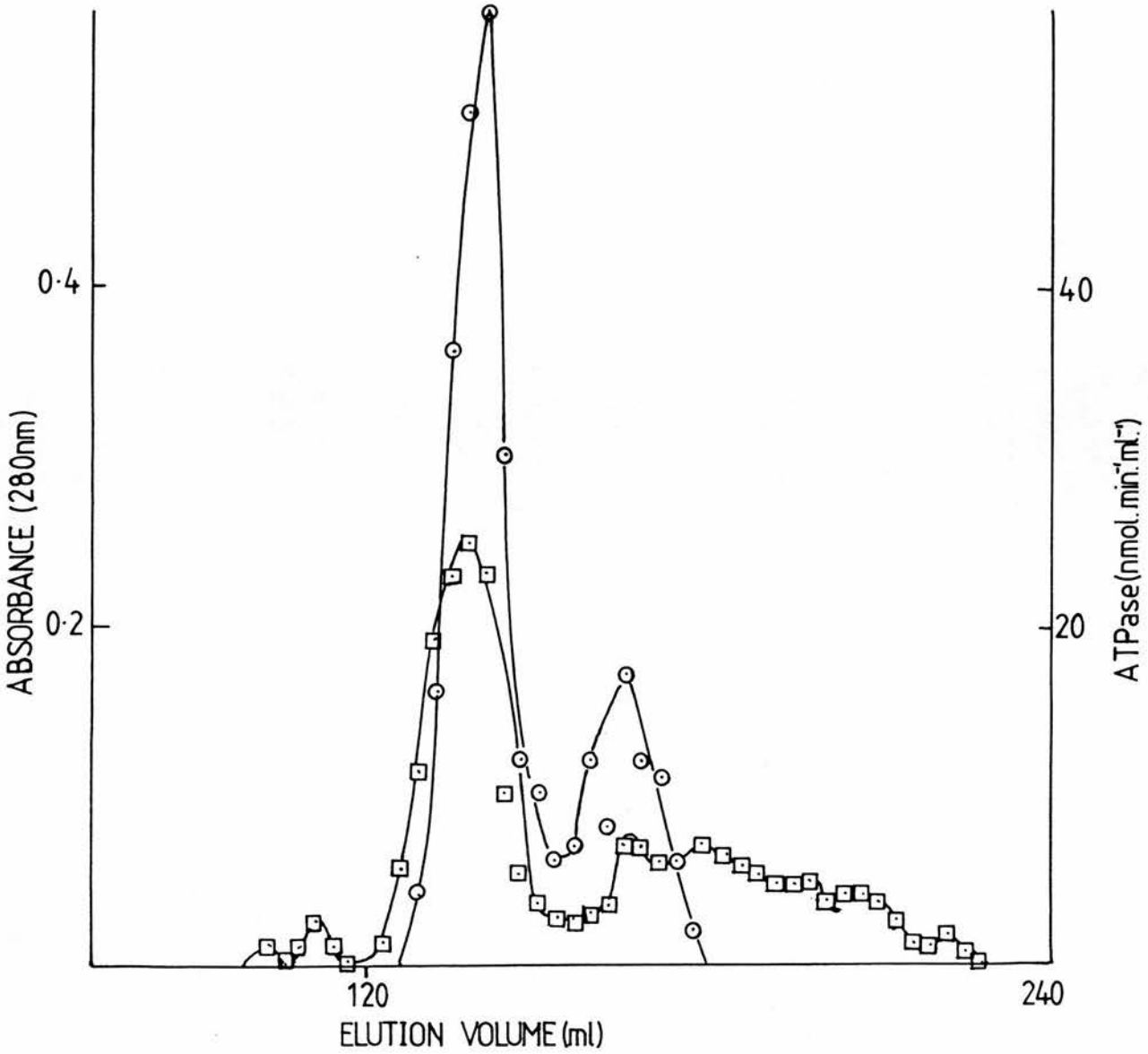


Figure 4.3. Elution profile of $C_{12}E_8$ solubilised chromaffin granule membranes on Sephacryl 300.

The figure shows ATPase activity (o) and absorbance at 280 nm (□) for $C_{12}E_8$ solubilised chromaffin granule membranes as described in Chapter 4.3.3.

fractions. Absorbances at 280 nm were measured and ATPase assays performed on those samples containing protein. The elution profile is presented in Figure 4.3. As can be seen from the profile of absorbance at 280 nm, about 50% of the protein eluted or near to the void volume. This could have been either due to protein aggregating in protein:lipid micelles and migrating as high molecular weight species of protein causing scattering or because 50% of the membrane protein exists in polymers of native weight greater than 400000. The ATPase could clearly be divided into two separate peaks. The major peak of activity (PEAK I) occurred just behind the first major protein peak, which eluted with the void volume. This gave a molecular weight of about 450000 for this peak, with the reservation made earlier. The first peak contained 66% of the total ATPase activity. The other 33% (PEAK II) eluted later at about a molecular weight of 150000. Whilst PEAK I is consistent with the idea of an F_oF_1 ATPase type of complex the second peak is more difficult to explain. The molecular weight of this peak is inconsistent with it being F_1 which has become detached from the proton channel (F_o) and as demonstrated later in this chapter this peak shows no reaction with antibodies raised against beef F_1 ATPase. Thus it may be that there is more than one type of ATPase in the chromaffin granule membrane. This is consistent with results presented in Chapter 3 which showed that ATPase activity was incompletely inhibited by DCCD. Finally the ATPase activity eluting from the column represented 90% of the total loaded. Thus there was some loss of ATPase activity.

4.3.4. Chromatography of $C_{12}E_8$ solubilised chromaffin granule membranes labelled with a low concentration of DCCD. The primary object of this experiment was to find out where the DCCD-reactive protein eluted from the column with respect to the ATPase activity.

Method. Freshly prepared chromaffin granule membranes (6 mg protein

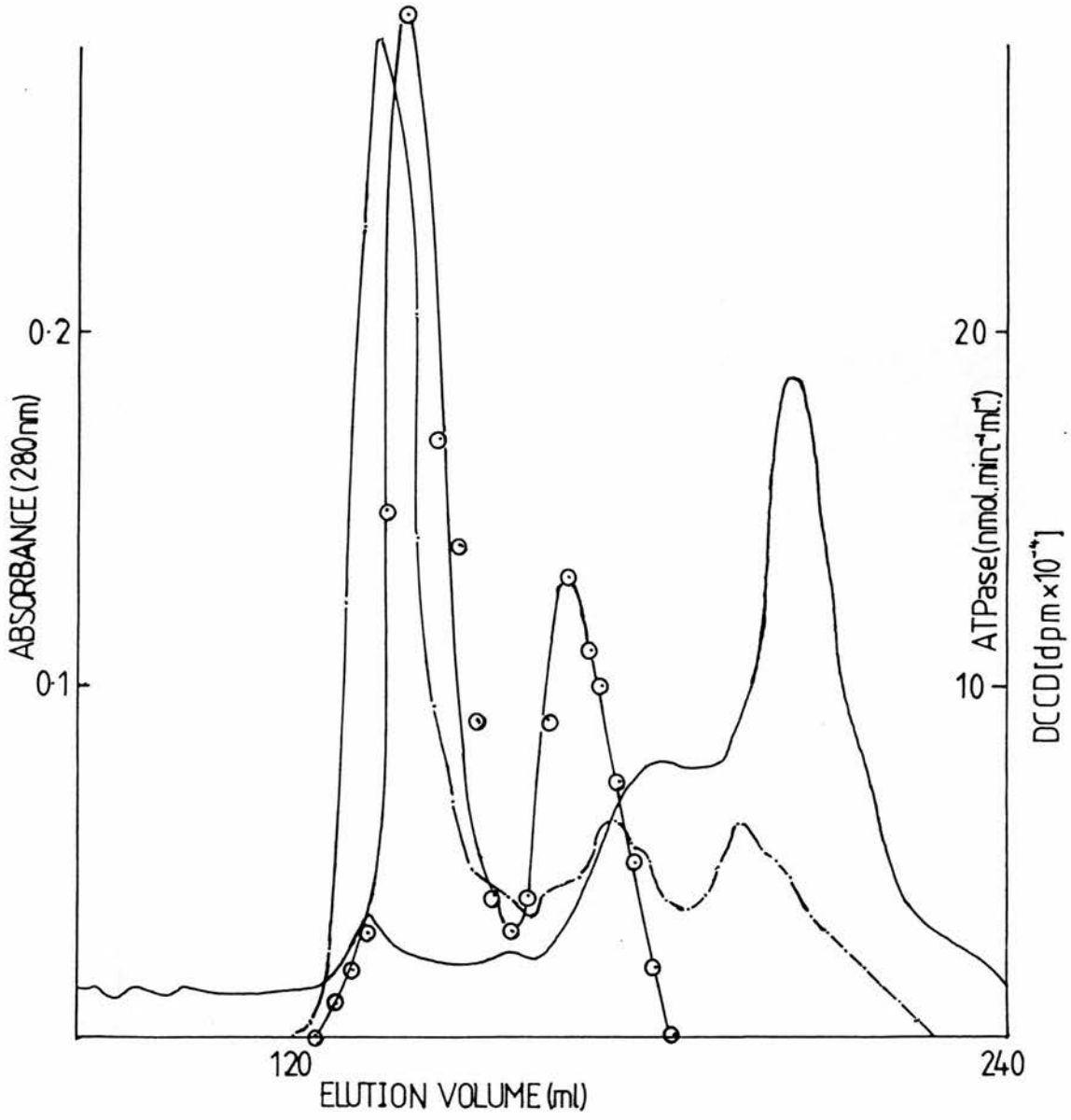


Figure 4.4. Elution profile of DCCD labelled $C_{12}E_8$ solubilised chromaffin granule membranes.

This figure shows absorbance at 280 nm (—·—), ATPase activity (⊙) and ^{14}C-DCCD (solid lines) elution pattern for $C_{12}E_8$ solubilised chromaffin granule membranes as described in Chapter 4.3.4.

	No DCCD	20 µM DCCD	% activity
Total absorbance (280 nm)*	1.92	1.88	
Total ATPase	891	546	62
Peak I ATPase	594	333	57.3
Peak II ATPase	297	213	73.5

Table 4.7. Relative inhibition of Peak I and Peak II ATPase activities of $C_{12}E_8$ solubilised chromaffin granule membranes inhibited with DCCD.

The figures presented are from the areas under the ATPase activity and protein elution profiles of $C_{12}E_8$ solubilised chromaffin granule membranes eluted from an S300 column as described in the text. The elution profiles used are presented in Figure 4.3 and 4.4.

* This was calculated from the areas under the 280 nm profile shown in figures 4.3 and 4.4, and are in arbitrary units

ml^{-1}) were labelled with 20 mM ^{14}C-DCCD in the presence of 10 mM ATP. After labelling, the membranes were washed by ultracentrifugation and resuspended at 8 mg protein ml^{-1} in Hepes buffer. At 30°C 5 mg of $C_{12}E_8$ per mg of protein was added dropwise from a 10% stock solution. The solution was centrifuged (50000 r.p.m. Beckman Ti50 rotor, 4°C, 30 mins g_{av} = 26000), 2 mls of the supernatant loaded onto the Sephacryl 300 column and eluted with column buffer as described in section 4.3.2. Absorbance at 280 nm, ATPase activity, and radioactivity elution profiles are presented in Figure 4.4.

The elution profile is very similar to that shown in Figure 4.3. However it appears that there is only a very small peak of radioactivity comigrating with PEAK I ATPase whereas most of the radioactivity elutes after the ATPase activity. Calculations based upon the ATPase activities are presented in Table 4.7. These results clearly show that there is a differential sensitivity to DCCD between the PEAK I and PEAK II ATPase activity. PEAK I appears to be 50% inhibited by DCCD treatment of the membranes, whilst PEAK II is only 25% inhibited by DCCD. The overall inhibition of the ATPase is somewhat higher than that observed in intact granules which may be due to the solubilisation procedure, or due to the subsequent chromatography of the membranes. The differential sensitivity to DCCD fits with the observation that there may be two ATPases in the chromaffin granule membrane. However it is still not clear whether PEAK II ATPase activity is due to detachment of F_1 ATPase from the membrane or to a different ATPase.

Gel electrophoresis of the fractions from this column are presented in Figures 4.5 (a)-(c). The DCCD-reactive protein appeared as a single peak which eluted with PEAK I ATPase. No DCCD-reactive protein eluted elsewhere on the column which presents strong evidence to suggest that PEAK II ATPase is not due to detachment of F_1 from the membrane, although

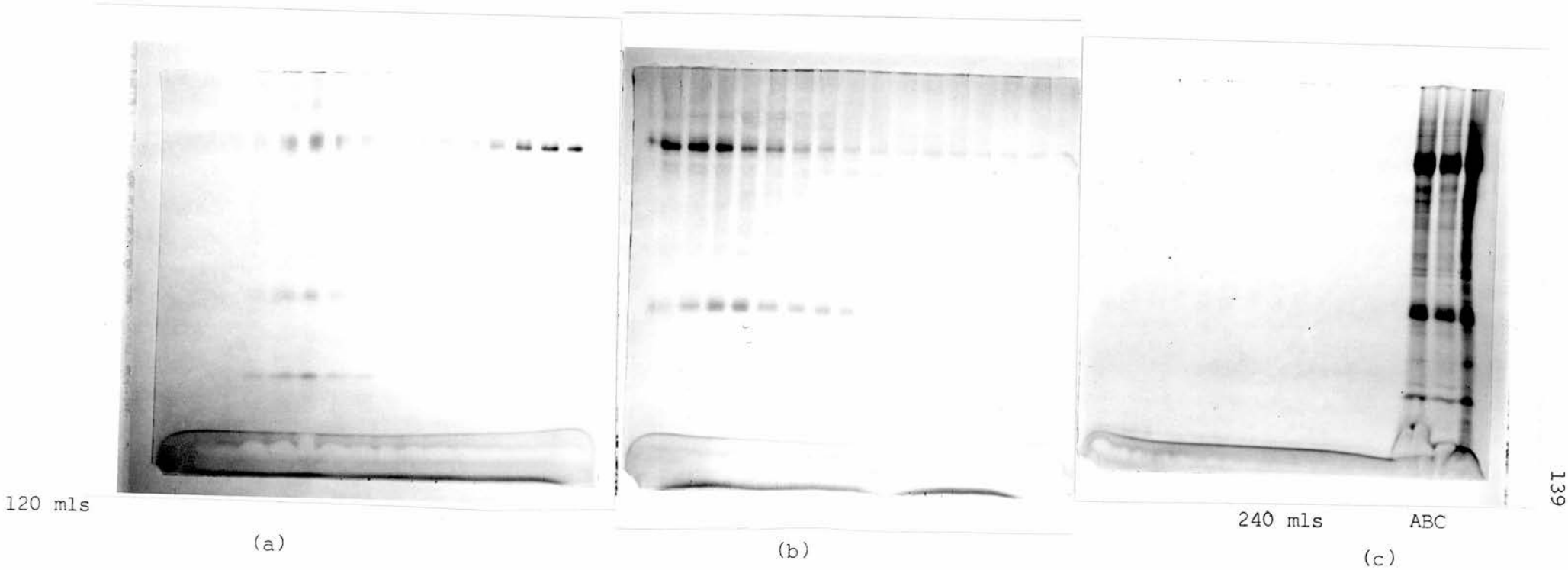


Figure 4.5. Elution of DCCD bound material from $C_{12}E_8$ solubilised ^{14}C-DCCD labelled chromaffin granule membranes.

(a)-(c) 10-15% exponential gradient SDS polyacrylamide gels of successive fractions from ^{14}C-DCCD labelled, and stained with Coomassie
$C_{12}E_8$ solubilised chromaffin granule membranes, electrophoresed as described in the text, A = supernatant; B = pellet; C = solubilised membrane. These fractions were taken from the experiment shown in fig. 4.4.

it may be rather difficult to solubilise Fo unless it is attached to F_1 ATPase (see also Chapter 7). The pellet from the centrifugation after the granule membrane was solubilised clearly shows that a reasonable proportion of the DCCD reactive protein remains unsolubilised, and it may be that only F_oF_1 complex is $C_{12}E_8$ soluble. The major peak of radioactivity can be seen to be due to the elution of lipids from the column. The shoulder on this peak is due to elution of an unusual protein which stretches from 80-120000 on polyacrylamide gels. The molecular weight of 85000 from the elution profile on the column suggests the protein may be a monomer within the membrane. Based upon the coomassie staining pattern of the gel this protein appears to be a major constituent of the membranes (see Abbs and Phillips, 1980). Furthermore it has been observed that catecholamine transport is inhibited by DCCD (Apps et al., 1980(b)). Conceivably this is the catecholamine transporter. If as is suggested in Chapter 1, catecholamines are exchanged electrogenically for protons, then the catecholamine transporter must have one or two proton binding sites and this may make the protein DCCD sensitive. DCCD has been shown to inhibit a number of proton linked transport activities (Casey et al., 1980, Sigrist-Nelson and Azzi, 1980).

4.3.5. Elution of $C_{12}E_8$ solubilised chromaffin granule membrane F_1 ATPase

Since it has been shown in section 4.3.4. that the DCCD-reactive protein elutes as a single peak, and that there are two ATPase peaks from gel chromatography of $C_{12}E_8$-solubilised membranes, it is obviously of interest to ascertain the elution pattern of F_1 like material.

Method. Freshly prepared chromaffin granules, were inhibited with DCCD solubilised with $C_{12}E_8$ and chromatographed on S300 as described in section 4.3.4. except that the column buffer contained 0.1% w/v

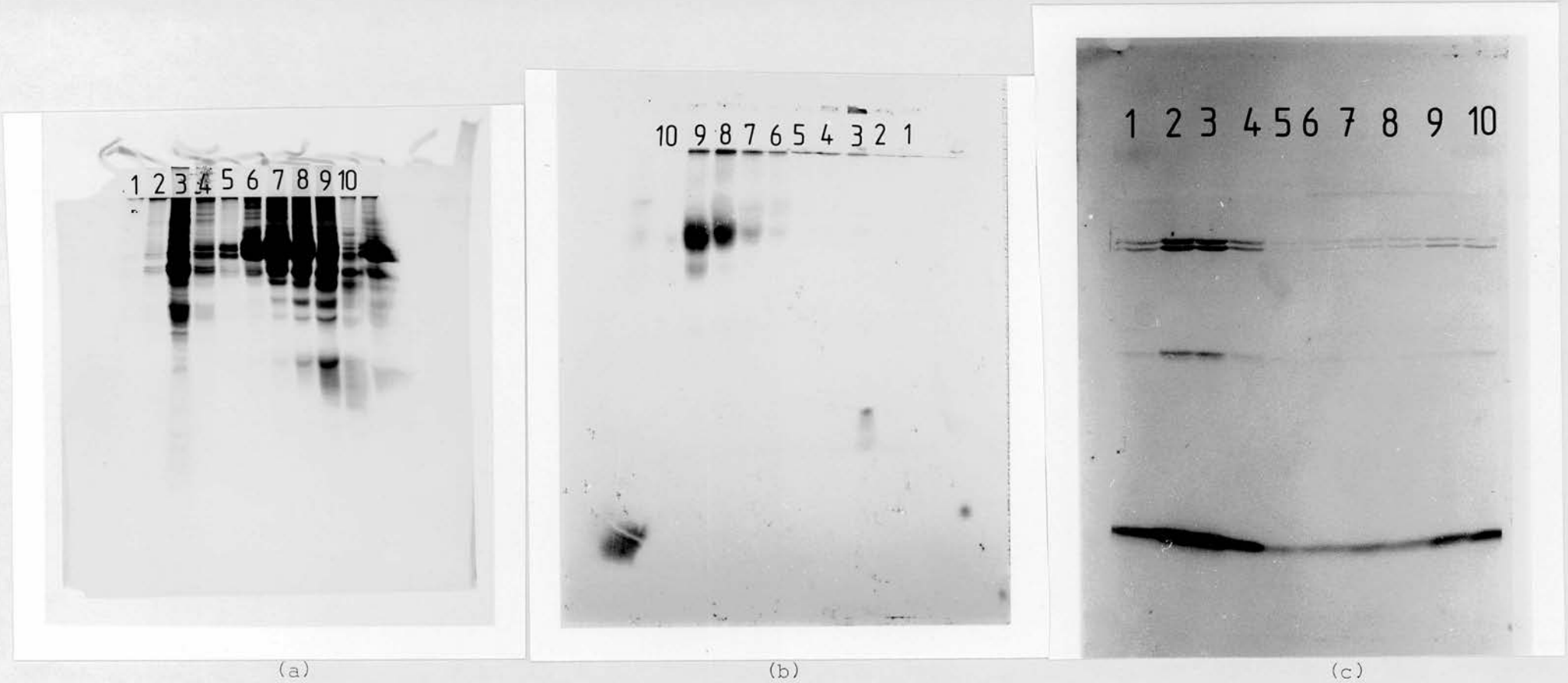


Figure 4.6. Elution profile of S300 chromatography of $C_{12}E_8$ solubilised chromaffin granule membranes to show pattern of anti beef F_1 ATPase.

(a) Staining pattern of 10-15% exponential gradient SDS polyacrylamide gel of pooled successive elute from ^{14}C-DCCD inhibited, $C_{12}E_8$ solubilised, chromaffin granule membranes. (b) Autoradiograph of (a). (c) Autoradiograph of cellulose nitrate sheets containing electrophoretically transferred proteins decorated with anti beef F_1 ATPase and subsequently with radioactive protein A.

$C_{12}E_8$ and 20% v/v glycerol. Samples containing protein were pooled into 9 ml fractions, dialysed to remove KCl against fifty volumes of distilled water at $0^{o}C$, and lyophilised. The fractions were dissolved in 1 ml of water and precipitated with 9 mls of acetone:ethanol 1:1 to remove lipid. The precipitates were dissolved in SDS sample buffer and subjected to SDS gel electrophoresis on two 10-15% exponential slab gels. One gel was treated with sodium salicylate and fluorographed as described in Chapter 2. The other gel was subjected to electrophoretic transfer of the proteins onto cellulose nitrate sheets and immune replica determination of anti beef F_1 ATPase binding proteins as described in Chapter 2. The cellulose nitrate replica was autoradiographed to show F_1 ATPase, the gel being subsequently stained for protein. The gel staining pattern of the fractions, the fluorograph of the gel to show ^{14}C and the autoradiograph of the immune replica are presented in Figures 4.6 (a)-(c).

The major point of interest with the elution profile is that most of the DCCD reactive protein elutes in fraction 3 with just a tiny amount eluting in fraction 4. The broad band protein (80-120000) elutes mainly in fractions 8 and 9. If one compares this to the coomassie blue pattern in 4(a) it is clear that this protein is distinct from either dopamine β-hydroxylase or chromogranin A both of which elute in fractions 6-8. The faint labelling seen in chromomembrin B also elutes in fractions 9 and 10. If one examines the staining pattern of fraction 3 it is noticeable that it is enriched in a number of specific bands, which do not elute in quantity elsewhere from the column. Examination of the immune replica of fractions reveals that the majority of the F_1 ATPase elutes in fractions 3 and 4, but there is a minor peak in fractions 10 and 11. The minor peak of F_1 ATPase eluting in fractions 10 and 11 is probably due to slow dissociation of the F_1 complex to

monomers and this would account for the very small traces of F_1 seen throughout the column.

The single peak of F_1 ATPase strongly suggests that the secondary peak of ATPase which would appear in fraction 6 is probably due to a second ATPase. It is perhaps of interest to note that cytochrome b_{561} elutes from the column with a peak in fraction 9 which gives it an estimated molecular weight of approximately 100000. Since Apps et al. (1980(c)) have shown that the SDS electrophoretic mobility of the protein is consistent with a molecular weight of 24000, this presents strong evidence that the cytochrome may function as a multimeric complex within the membrane which is consistent with results presented elsewhere (Silsand and Flatmark, 1974).

4.3.6. Chromatography of Taurodeoxycholate Solubilised Chromaffin Granule Membranes

More recently solubilisation of chromaffin granule membranes accompanied by complete separation of the two ATPase activity peaks upon S300 chromatography has been achieved using the zwitterionic detergent taurodeoxycholate (Apps, D.K. personal communication) and it is obviously of interest to compare the elution profile of the DCCD-reactive proteins and F_1 ATPase in an experiment analogous to experiment 4.3.5.

Method. Chromaffin granule membranes freshly prepared as described in section 2.1 were suspended at 12.15 mg protein ml^{-1} and 20 µM ^{14}C-DCCD added. The membranes were incubated at $4^{o}C$ for 16 hours in the presence of 10 mM ATP. To 530 µl of this suspension were added 1100 µl of distilled water, 250 µl 1M Hepes NaOH pH 7.4, 7 µl, 0.2 M EDTA pH 7.0, 12.5 µl 0.2 M DTT, 500 µl glycerol. After five minutes 75 µl 10% w/v taurodeoxycholate was added, the suspension thoroughly mixed

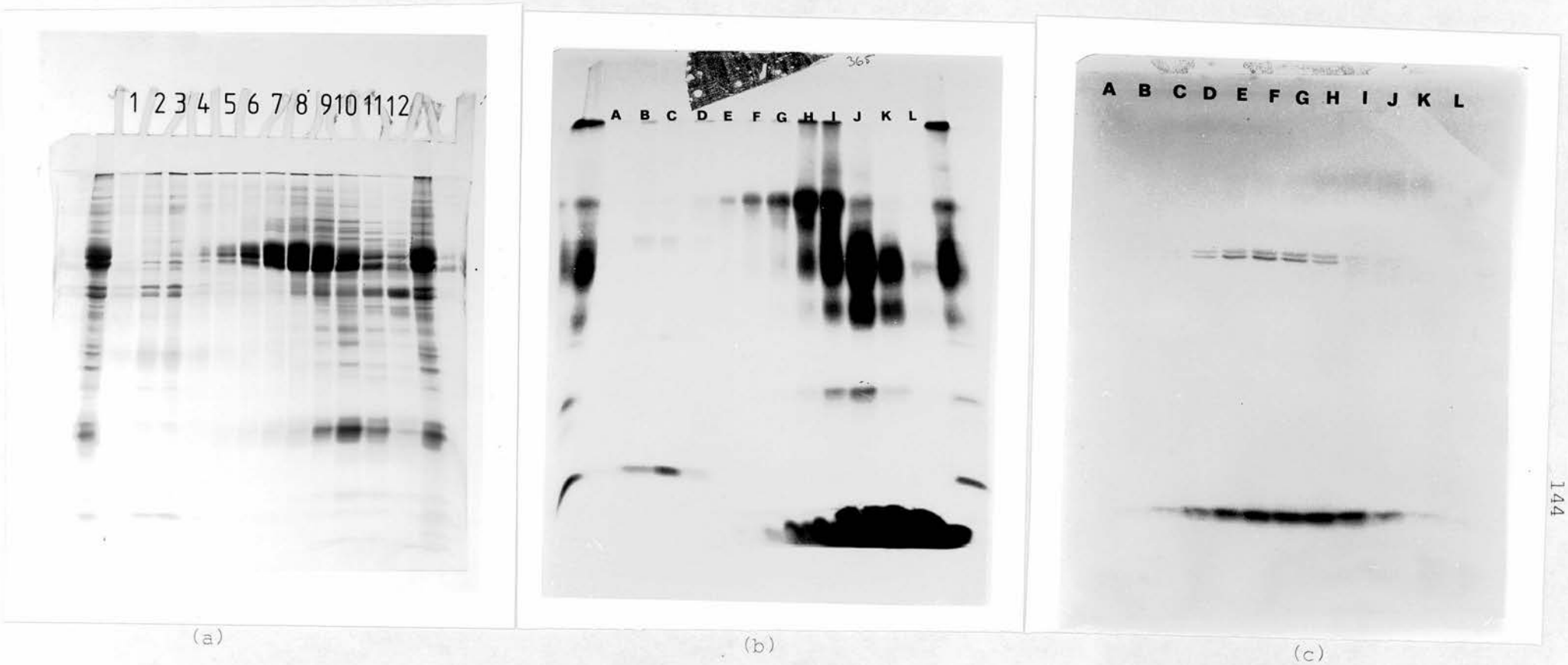


Figure 4.7. Elution profile of S300 chromatography of taurodeoxycholate solubilised chromaffin granule membranes to show pattern of anti beef F_1 ATPase.

(a) Staining pattern of 10-15% exponential gradient SDS polyacrylamide gel of pooled successive elute from ^{14}C-DCCD inhibited, taurodeoxycholate, chromaffin granule membranes. (b) Autoradiograph of (a). (c) Autoradiograph of cellulose nitrate sheets containing electrophoretically transferred proteins decorated with anti beef F_1 ATPase and subsequently with radioactive protein A.

loaded onto the column and eluted as described in section 4.3.6. Centrifugation of the solubilised membranes was found to be unnecessary as there was no visible pellet after such treatment. Electrophoresis, fluorography and immune replica were carried out as described in section 4.3.6. The stained gel, fluorograph to detect ^{14}C and autoradiograph of the immune replica to detect F_1 ATPase are presented in Figures 4.7 (a)-(c).

From the autoradiograph it can be seen that the low molecular weight protein which binds DCCD elutes in fractions B, C and D and this is matched by a heavily staining low molecular weight band in the stained gel. The early elution suggests that the DCCD-reactive protein is part of a high molecular weight complex which is about 450000 in molecular weight. The rest of the ^{14}C-DCCD radioactivity profile is very similar to that observed using $C_{12}E_8$; that is dopamine β-hydroxylase elutes in fractions G-I, the 80-120000 band eluted at fraction J (10) as does the cytochrome b_{561}. However the lipid which elutes in fractions I-L is later than that of the $C_{12}E_8$ solubilised membranes. This is probably due to the formation of small micelles using taurodeoxycholate.

The most interesting point in the immune replica is that F_1 ATPase elutes peaking at fraction F. This is considerably later than the DCCD-reactive protein. This could be either because taurodeoxycholate brings about separation of F_1 ATPase from the F_o fraction or that the DCCD-reactive protein is not associated with an F_1 like protein. It must be noted that taurodeoxycholate is the only bile salt which brings about an activation of the ATPase whilst the others inactivate it. This could be due to splitting of F_1 ATPase from the membrane with a concomitant increase in ATPase activity. Although the result could also be explained by the fact that there is nonspecific aggregation of proteins in the $C_{12}E_8$ solubilised membranes, whilst taurodeoxycholate, because of its

different properties does not give rise to large micelles. The arguments presented here favour the former theory of taurodeoxycholate dependent separation of F_o from F_1 ATPase. Finding the pH optimum of ATPase activity peaks eluting from the S300 gel could lead to more conclusive evidence about the effects of taurodeoxycholate on the solubilisation of chromaffin granule ATPase.

4.4. Summary

1. A wide range of detergents has been used to solubilise chromaffin granule membranes, and $C_{12}E_8$ has been found to be best at solubilising an active ATPase complex.

2. Best conditions for solubilising membranes were found to be high temperatures (25-30^{o}C) and low salt concentration (10 mM Hepes).

3. Two stage solubilisation of the membranes has been tried, and although some success has been found, the two stage solubilisation procedure leads to no better ATPase activity than a one stage solubilisation procedure.

4. Chromatography on Sephacryl S300 molecular exclusion column of detergent solubilised membranes gives clear evidence of assocation of the low molecular weight DCCD-reactive protein with an F_1-like ATPase.

5. Chromatography reveals the presence of a labelled protein whose molecular weight was ascertained to be 85000 by gel chromatography. Evidence is also presented for a polymeric form of cytochrome b_{561}.

6. Chromatography shows the presence of a low molecular weight ATPase M_r = 150000 (PEAK II ATPase) which is not due to F_1-like ATPase, and which shows a low sensitivity to DCCD when compared to the PEAK I ATPase.

Chapter 5

Purification of chromaffin granule membrane DCCD-reactive protein

Source of Protein	Prewash membranes	Extraction of the DCCD-reactive protein	Precipitation of DCCD-reactive protein	Further purification	References
(a) Mitochondria					
Ox heart) 1. Beef heart)	-	20 vols. $CHCl_3$: CH_3OH, 2:1	4 vols ether	1. H_2O wash 2. LH20 chromatography	Cattell et al. (1971) Steckhoven et al. (1972)
2. Beef heart		25 vols CH_3Cl_3: CH_3OH, 2:1	4 vols ether	1. H_2O wash 2. DEAE chromatography 3. LH chromatography	Graf and Sebald (1978)
1. Rat liver a)	-	9 vols $CHCl_3$:CH_3OH, 2:1	-	H_2O wash	) Burke and Beattie (1973)
b)	9 vols CH_3OH	2 vols $CHCl_3$:CH_3OH, 2:1	-	-	)
2. Rat liver		50 vols n-butanol	5 vols ether	-	Sigrist et al. (1977)
Mouse liver	10 vols CH_3OH: $CHCl_3$:ether 1:2:12	20 vols $CHCl_3$:CH_3OH, 2:1			Aroskar and Avadhani (1980)
S. cerevisiae	9 vols CH_3OH	3 vols $CHCl_3$:CH_3OH, 2:1	4 vols ether	TLC	Sierra and Tzagaloff (1974) Sebald et al. (1979)
N. crassa	-	20 vols $CHCl_3$:CH_3OH, 2:1	3 x 4 vols ether	-	Sebald et al. (1979)
(b) Chloroplast					
Lettuce		50 vols n-butanol			Nelson et al. (1978) Sigrist-Nelson et al. (1979)
(c) Plasma membrane					
E. coli		25 vols $CHCl_3$:CH_3OH, 2:1	4 vols ether	1. H_2O wash 2. DEAE chromatography 3. LH60 chromatography	Fillingame (1975, 1976)
PSIII	vols 50% NaOH in 10 mM $MgSO_4$	vols $CHCl_3$:CH_3OH, 3:2	ether	-	Sone et al. (1978)

Table 5.1. Purification of low molecular weight DCCD-reactive proteins from various sources.

5.1 Introduction

It has been shown in previous chapters that chromaffin granule membranes contain a low-molecular weight protein which binds DCCD covalently and evidence has been presented which suggests that it is part of an ATPase complex. The present chapter deals primarily with the purification of this protein, using ^{14}C-DCCD as a marker, and with stoichiometric aspects of the ATPase complex. Similar proteins have been isolated from a number of different sources. In the earliest attempts at isolation of such a protein, using ox heart mitochondria, Cattell et al. (1971) extracted the protein into chloroform:methanol (2:1 unless otherwise stated) using enough solvent to give a single phase. Since this solvent also extracts lipids from the membrane, proteins thus extracted have been termed proteolipid (Folch and Lees, 1951). However, the term proteolipid implies some covalent modification of the protein by lipid. For the purposes of this text the terms low molecular weight organic solvent soluble protein, or DCCD-reactive protein shall be used to describe these proteins, as no covalent modification of amino acid side chains by lipid has yet been demonstrated in any of these proteins.

The rest of the purification procedure is described briefly in Table 5.1. Also shown in Table 5.1 are the different purification procedures by several different authors to purify the DCCD-reactive proteins from various sources. There are some generalisations that can be drawn from Table 5.1. At some stage of the purification, the DCCD-reactive protein is extracted into an organic solvent, which also extracts lipids. The solvents so far used are chloroform:methanol (either 2:1 or 3:2) and butanol, although the chloroplast DCCD-reactive protein was found to be soluble in a number of different organic solvents (Sigrist-Nelson et al., 1978). In other stages of the purification the

DCCD-reactive protein was separated from extracted lipid, by extraction of the lipids into a solvent in which the proteolipid was insoluble. Further purification, when necessary, used either ion exchange or molecular exclusion, gel chromatography and thin layer chromatography.

5.2.1 Attempts to extract the DCCD-reactive protein

In this experiment an attempt was made to purify the chromaffin granule DCCD-reactive protein by extraction of the protein with chloroform:methanol, followed by precipitation with diethyl ether. As a control, to check the method, beef heart mitochondria were used in an identical purification procedure.

Method. 1.0 ml of pure chromaffin granule membranes (4.7 mg protein.ml^{-1}) were labelled in the presence of 5 µM ^{14}C-DCCD. Also ^{14}C-DCCD-labelled mitochondria (8.6 mg protein ml^{-1}) were used in an identical procedure. To each membrane solution 25 mls of freshly prepared chloroform:methanol was added and the mixture incubated at $0^{o}C$ for 12-16 hours. After this time the solution was centrifuged (20 mins, 20 000 rpm, $4^{o}C$ Beckman JA20 rotor, g_{av} = 18 000) in glass tubes to precipitate insoluble material. The supernatants were carefully decanted, and the pellet stored. To the supernatants 6.25 mls of twice-distilled water was added, and the mixtures vigorously shaken for 20 s, before being left to separate in a separating funnel at room temperature (about twelve hours). After this time the bottom layers were removed and the top layers stored. The bottom layers were rotary-evaporated to dryness and resuspended in 5 mls of chloroform:methanol. To the chloroform:methanol, 20 mls of diethyl ether were added, and the solutions left to cool to $-15^{o}C$ for twelve hours. The solutions were then centrifuged at $-10^{o}C$ as before. The supernatants were carefully decanted and stored, the precipitates being kept in chloroform:methanol

Table 5.2.(a)

Sample	d.p.m. x 10^{-2}	Volume (mls)	Total d.p.m.	Fraction of radioactivity %
chromaffin granule membranes	6734	5.7	3838380	100
chloroform/methanol supernatant	145	188	2726000	71
chloroform/methanol precipitate	600	8.6	516000	33.4
water soluble layer	0	95	0	0
chloroform soluble layer	96	132	1267300	33
ether supernatant	9990	1.3	1298700	34
ether precipitate	39	3.15	12285	0.3

Table 5.2.(b)

mitochondrial membranes	6811	5.9	4018490	100
chloroform/methanol supernatant	147	192.5	2829750	70.4
chloroform/methanol precipitate	506	7.9	399740	9.9
water soluble layer	4	92	36800	0.9
chloroform soluble layer	165	136	2234000	56
ether supernatant	579	46	2663400	66.3
ether precipitate	161	2.5	100625	2.5

Table 5.2. ^{14}C-DCCD distribution during organic solvent solubilisation of the membrane.

(a) Shows the amount of ^{14}C in each fraction during the attempted purification of the DCCD-reactive protein described in Chapter 5.2.1.

(b) As in (a) except for mitochondrial membranes.

in which they were soluble. Samples (10 µl) were taken at each stage for scintillation counting, and (100 µl) for polyacrylamide gel electrophoresis. Fractions that were in organic solvent were dried down in a stream of nitrogen before redissolving in SDS-containing sample buffer for polyacrylamide gel electrophoresis.

Results. The samples which were kept for scintillation counting were dried onto small squares (1 cm^2) of cellulose nitrate paper and then added to 2.5 mls of toluene scintillant. Volumes at each stage of the extraction procedure were noted and the results are presented in Table 5.2. From this table several things can be noted. First, a much larger percentage of the counts (some eight hundred) were found in the ether precipitate of the mitochondrial extract than in the same fraction in the chromaffin granule purification. This could be due to a larger proportion of DCCD-reactive protein in mitochondria extracting into chloroform:methanol, a higher percentage of the mitochondrial protein per unit milligram of membrane, as suggested by the labelling experiments, or a larger fraction of the mitochondrial protein binding DCCD. Second, only a small percentage of the total counts appear in the ether precipitate in contrast to similar reported extractions (Sigrist-Nelson and Azzi, 1979; Cattell et al., 1971). This could be due to the fact that a large excess of labelled DCCD was added to both chromaffin granule and mitochondrial membranes in the initial incubation whereas to the previously published results involved the use of just enough ^{14}C-DCCD to maximally inhibit the ATPase activity. Third, a large fraction of radioactivity added to the chromaffin granule membrane remains in the protein precipitated during the chloroform:methanol extraction. Reasons for this could be either incomplete extraction of the DCCD-reactive protein or the effect that ^{14}C-DCCD binds several other membrane components which are insoluble in chloroform:methanol (see

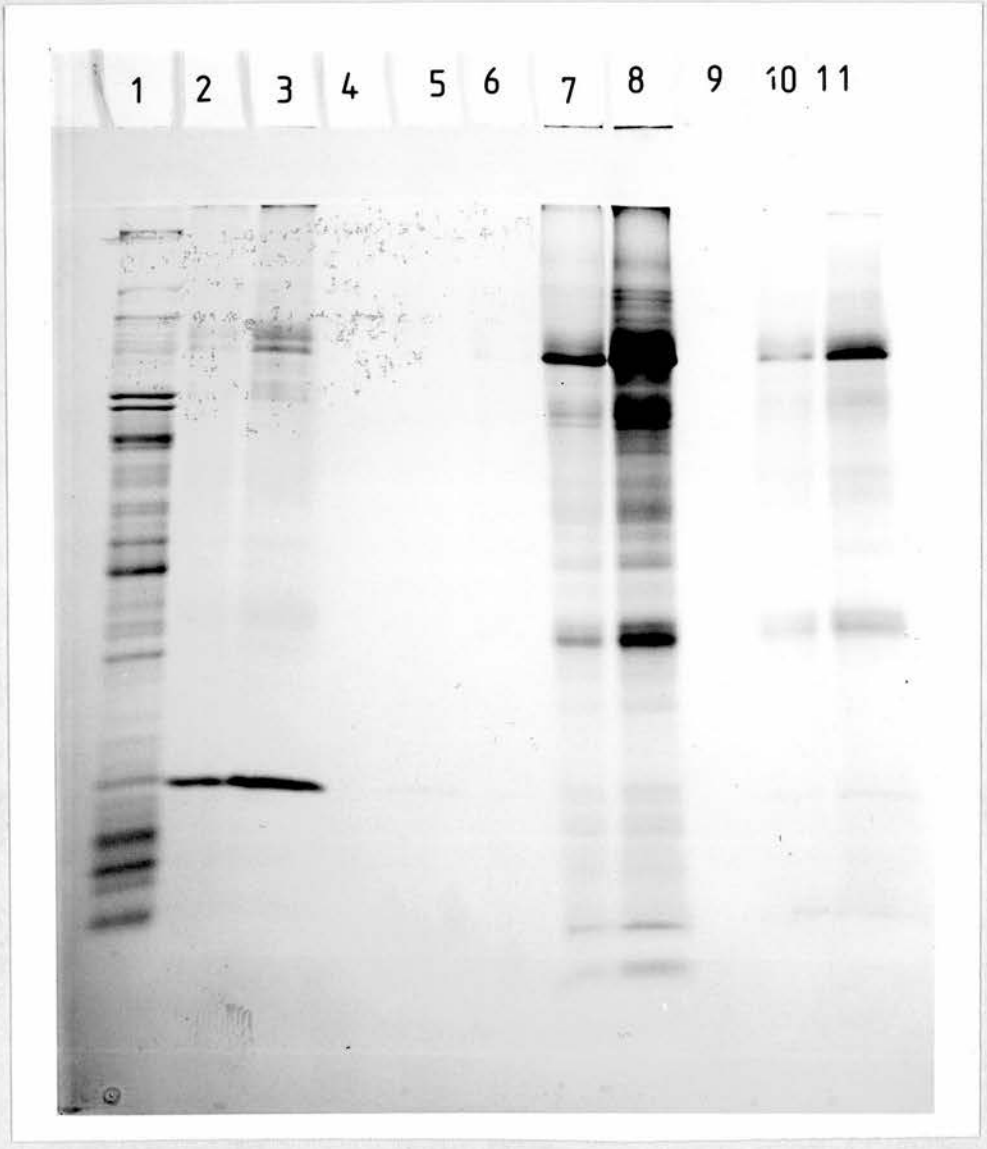
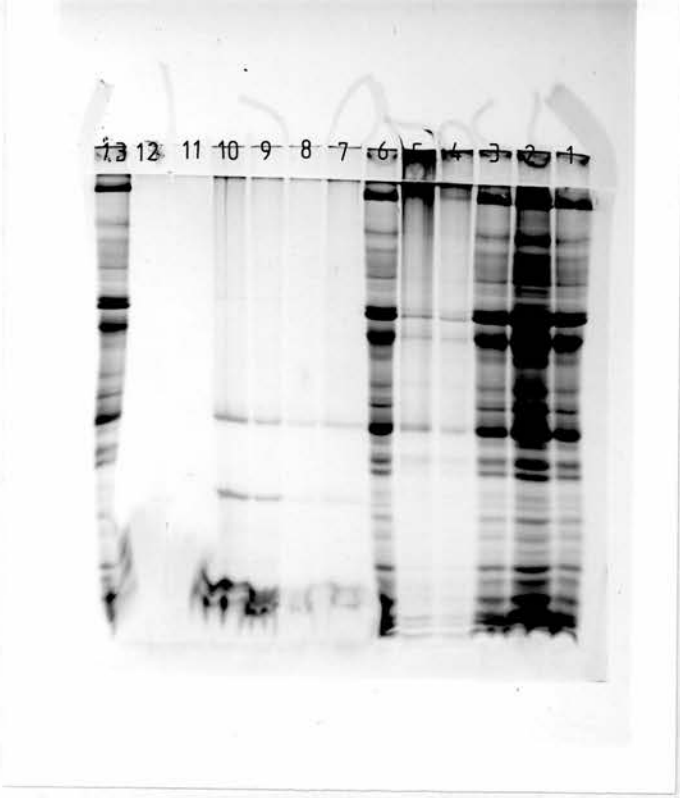


Figure 5.1. (a) Attempted purification of the DCCD-reactive proteins from chromaffin granules and adrenal mitochondria.

10-15% exponential gel of fractions during the initial attempt to purify the chromaffin granule DCCD reactive protein. TRACKS 10 and 11, chromaffin granule membranes; 7 and 8, chloroform:methanol precipitate; 4 and 5, ether supernatant; 2 and 3, ether precipitate; 1, mitochondrial membranes.



(b)

(c)

Figure 5.1. (b) and (c). Attempted purification of the DCCD-reactive proteins from chromaffin granules and adrenal mitochondria.

(b) 10-15% exponential gradient SDS polyacrylamide gel of fractions from the adrenal mitochondrial DCCD-reactive protein purification. TRACKS 1, 2, 3, 6 and 13, mitochondrial membranes (^{14}C-DCCD labelled); 4 and 5, chloroform: methanol precipitate; 7 and 8, chloroform:methanol supernatant; 9 and 10, ether precipitate; 11 and 12, ether supernatant.

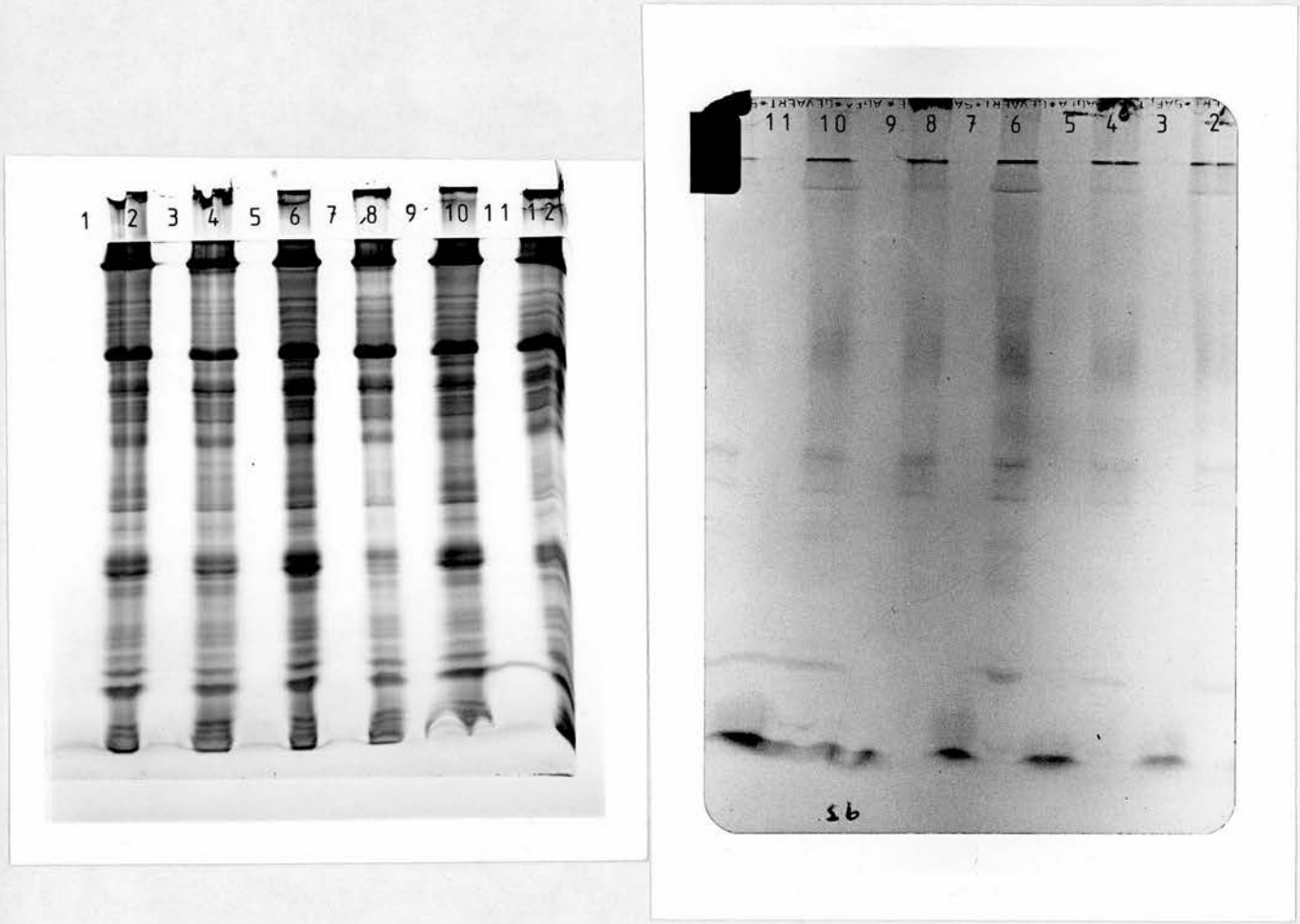
(c) Autoradiograph of gel in (b).

Fig. 3.11).

SDS polyacrylamide gel electrophoresis of the fractions from this purification can be seen in Fig. 5.1(a)-(c). A mitochondrial component which is labelled by ^{14}C-DCCD but which is poorly stained in the gel, is extracted by chloroform:methanol and precipitated by diethylether. Although a protein was extracted by chloroform:methanol from chromaffin granules, there was no indication that this protein was radioactively labelled, even after twelve weeks autoradiography of the dried gel. Nor did slicing of bands from the gel followed by scintillation counting reveal any sign of a DCCD-reactive component in the extract. Another point to note was the highly variable nature of the extraction procedure. Several attempts to repeat the chromaffin granule results proved unsuccessful and even unlabelled protein was not extracted. Furthermore difficulties were encountered in maintaining a low enough temperature during the ether precipitation of extracted material, leading to incomplete precipitation of extracted material at this stage. Why did this extraction procedure not purify labelled protein? Apart from the reasons already mentioned could be the simple fact that there was too little incorporation of ^{14}C-DCCD into the protein as the phenomenon of ATP enhanced DCCD binding to the membranes had not been discovered at the time. Also fluorography with salicylate had not been published at the time of these experiments. Finally the relatively high-proportion of lipid:protein in the chromaffin granule membranes when compared to mitochondria may have hindered the subsequent ether precipitation steps.

5.2.2 Solvent solubility of the DCCD-reactive protein

In order to obtain a basis for the purification of any DCCD-reactive protein a preliminary screening of a number of lipid solvents



(a) (b)

Figure 5.2. Solvent solubility of the chromaffin granule DCCD-reactive protein

(a) 10-15% exponential gradient SDS-polyacrylamide gel of ^{14}C-DCCD labelled chromaffin granule membranes which had been treated by organic solvent extraction as described in the text. Odd tracks show solvent soluble material, even tracks solvent insoluble material. TRACKS 1 and 2, methanol; 3 and 4, ethanol; 5 and 6, propanol; 7 and 8, butanol; 9 and 10, acetone; 11 and 12, chloroform:methanol.

(b) Autoradiograph of gel in (a).

was undertaken. The solvents chosen were based on the observation that the corresponding protein from chloroplasts was soluble in a number of different organic solvents (Sigrist-Nelson et al., 1978).

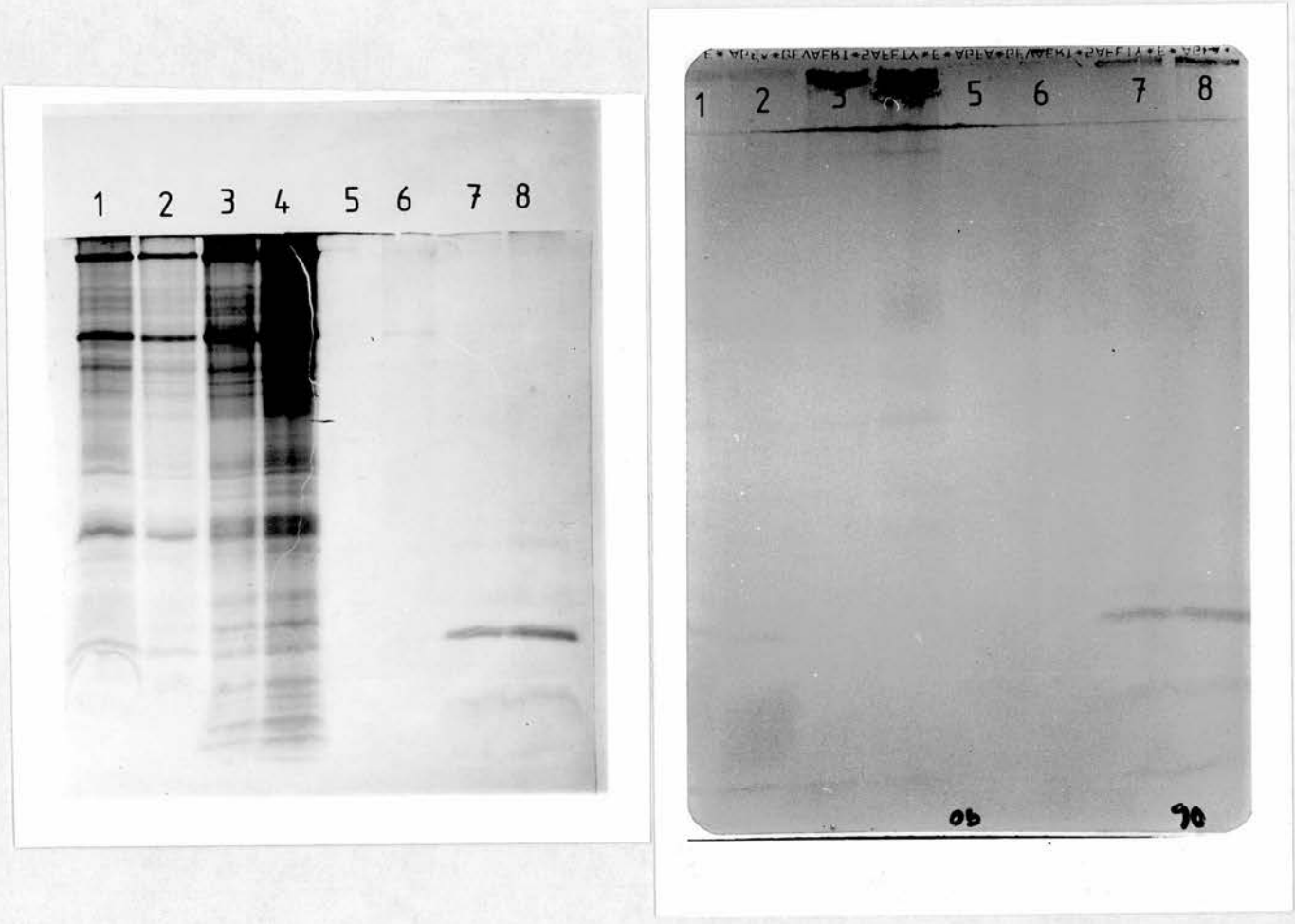
Method. Chromaffin granule membranes (4.8 mg protein ml^{-1}) which had been labelled with ^{14}C-DCCD in the presence of ATP (see Chapter 3) were washed by ultracentrifugation and resuspension in Hepes buffer. 1 ml aliquots of this suspension were added to 25 mls of methanol, ethanol, propanol, n-butanol, acetone and chloroform:methanol, and left slowly stirring for 16 hours at $0^{o}C$. Each mixture was then centrifuged to remove insoluble material. The supernatants were carefully decanted, filtered through glass wool, rotary evaporated to dryness, and dissolved in SDS-containing sample buffer for SDS-polyacrylamide gel electrophoresis. The pellets of each fraction were also dissolved in SDS for gel electrophoresis. 100 µl samples were electrophoresed on 10-15% exponential polyacrylamide gels, fixed, stained, destained and dried. The dried gel was autoradiographed for four weeks. The result of this experiment can be seen in Figure 5.2 (a) and (b).

The only organic solvent to extract labelled protein was the chloroform:methanol mixture although butanol has been subsequently reported to extract the labelled band in impure form (L.M. Wakefield, personal communication). This is in contrast with the results presented here. The step in which the supernatant was filtered prior to rotary evaporation removed the small amount of higher molecular weight contaminants that had previously been seen (Fig. 5.1(a) tracks 2 and 3) in SDS polyacrylamide gels. However the problem of separating protein from the lipid-like material remained. From Fig. 5.2 it can be seen that most of the other organic solvents used, whilst not solubilising the proteolipid, removed large amounts of residual phospholipid. Thus

SAMPLE	d.p.m./ml x 10^{-2}	Volume (ml)	Total d.p.m. x 10^{-2}	Fraction of radioactivity %
Starting membranes	19085	1	19085	100
Acetone precipitate	341	1	341	1.8
Acetone supernatant	761	24.66	18720	98.3
Chloroform/methanol supernatant	38	3	114	0.6
Chloroform/methanol precipitate	223	1	223	1.2

Table 5.3. Distribution of radioactivity during two stage purification of the chromaffin granule DCCD-reactive protein

The table shows ^{14}C at each stage of the purification procedure described in Chapter 5.2.3.



(a) (b)

Figure 5.3. Two stage purification of chromaffin granule membrane DCCD-reactive protein.

(a) 10-15% exponential gradient SDS-polyacrylamide gel of fractions during the purification of chromaffin granule membrane DCCD-reactive protein from ^{14}C-DCCD labelled chromaffin granule membranes described in Chapter 5.2.3. TRACKS 1, chromaffin granule membranes; 2, acetone insoluble material; 3 and 4, chloroform:methanol precipitate; 5 and 6, acetone solution; 7 and 8 chloroform:methanol solution.

(b) Autoradiograph of (a).

any of these solvents would be suitable for removal of lipid as a first stage in the purification; acetone was chosen.

5.2.3 Pre-extraction of lipid from chromaffin granule membranes

Membranes which had been labelled with ^{14}C-DCCD in the presence of ATP, were washed and resuspended in Hepes buffer as previously described. 1 ml of washed membranes were mixed with 25 mls of acetone at 0°C, and after 30 mins, centrifuged to remove insoluble material. The precipitate was resuspended in 1 ml of twice-distilled water, whilst the supernatant was rotary evaporated to dryness and stored. The precipitate was added to 25 mls of ice cold chloroform:methanol and left stirring slowly for 12-16 hours at 4°C. The solution was then centrifuged to remove insoluble material and the supernatant carefully decanted through a glass wool filter. The supernatant was then rotary evaporated to dryness. Samples from each stage were taken for scintillation counting as well as for SDS polyacrylamide gel electrophoresis. Results of this experiment can be seen in Table 5.3 and Figure 5.3(a) and (b).

There are several points of note from this experiment. The polyacrylamide gel shows that, although most of the lipid-like material is removed by washing the membranes with acetone, lipid extraction is not complete, and this material remains a contaminant of the protein when later extracted into chloroform:methanol. Also the table of results shows that in the acetone-insoluble material, only 1.8% of the total counts are retained, whilst the remainder is in an unbound, or lipid-bound form and is extracted into acetone. Thus acetone washing has the advantage that it removes most of the radioactivity which is not protein-bound (see also autoradiograph, Fig. 5.3(b)). Of the radioactivity in the acetone precipitate only one-third is soluble in

chloroform:methanol, whilst the remainder remains insoluble. This remainder probably corresponds to the higher molecular weight proteins that also become labelled with ^{14}C-DCCD as discussed in more depth in Chapter 3.

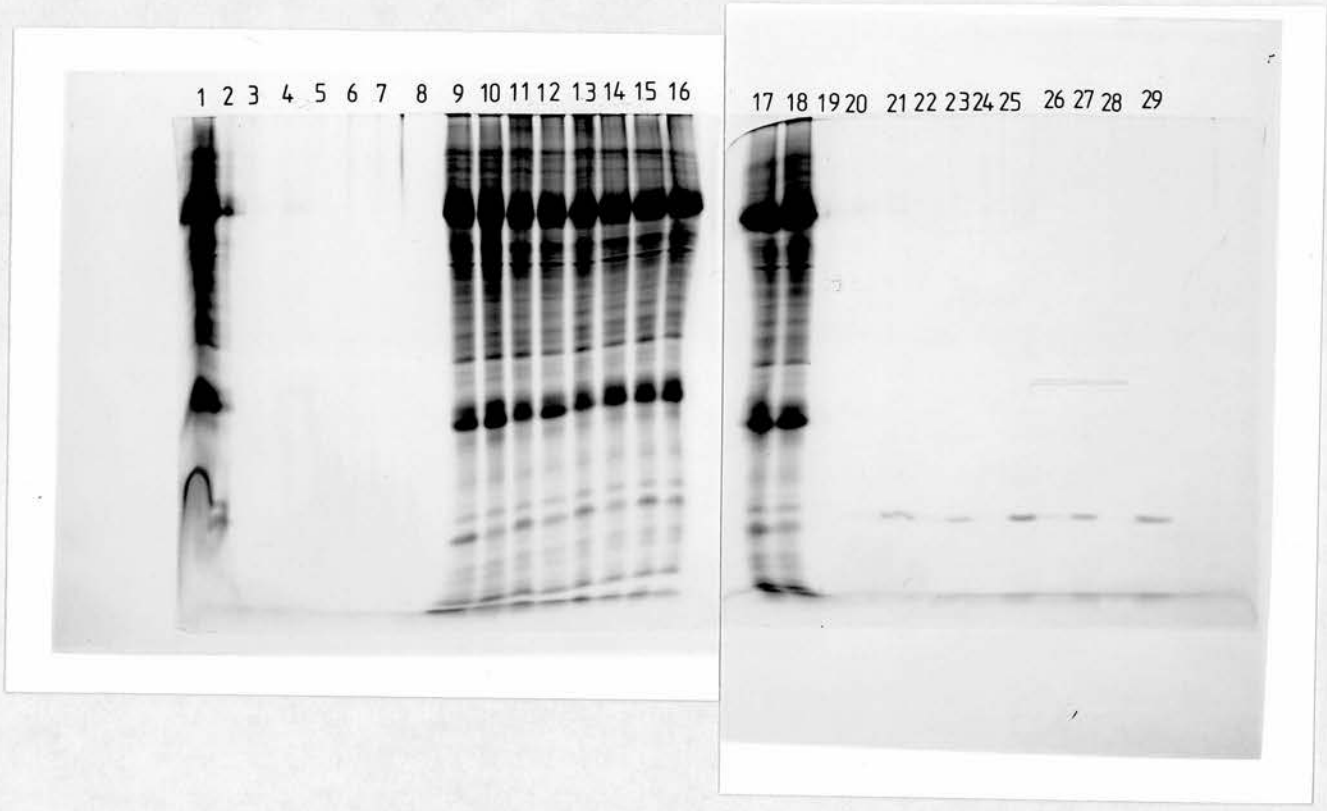
5.2.4 Optimization of lipid removal conditions

It would be of considerable advantage in the purification of the protein if the pre-extraction with organic solvents resulted in a more complete removal of lipids from the membrane, if this could be achieved without seriously affecting the solubility of the DCCD-reactive protein in chloroform:methanol. In this experiment, therefore, various mixtures of acetone and ethanol were used in the lipid extraction.

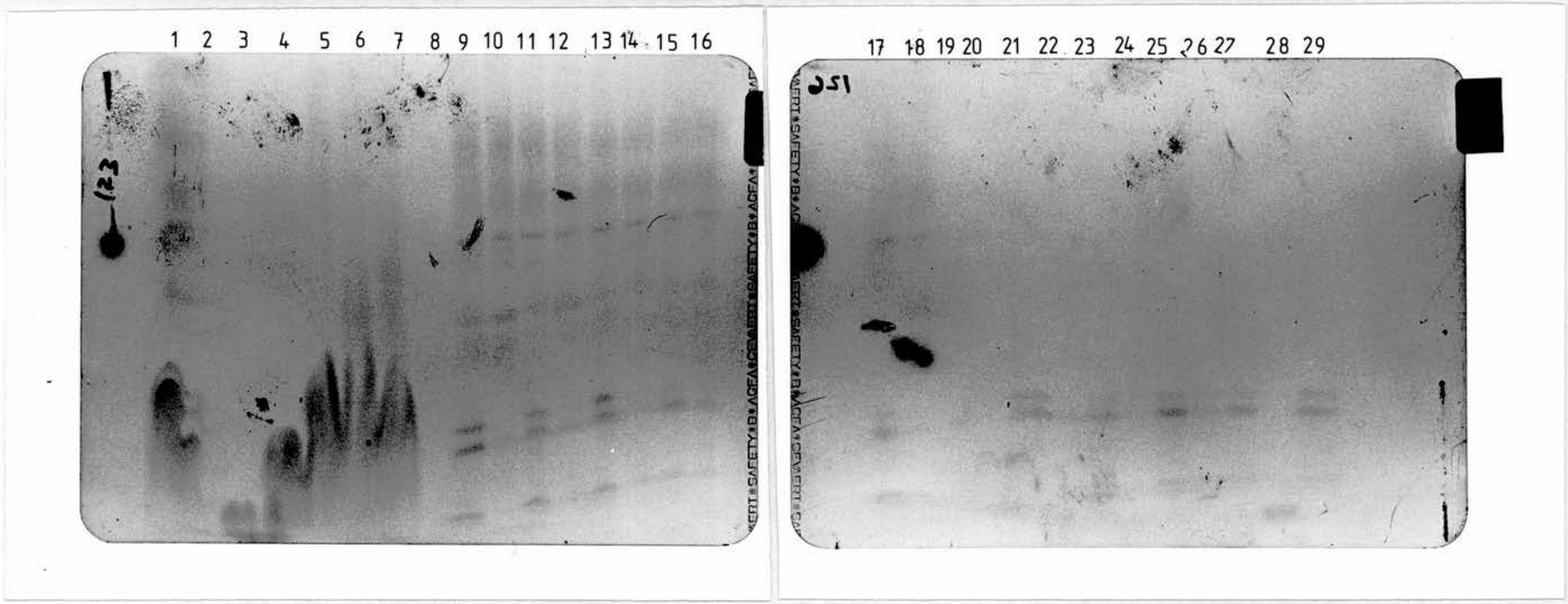
Method. Chromaffin granule membranes, labelled with ^{14}C-DCCD in the presence of ATP were washed by ultracentrifugation with Hepes buffer. Aliquots of 1 ml of the resuspended membranes were taken and added to 25 mls of the following solvents; acetone, acetone:ethanol (3:1, 1:1, 1:3) and ethanol (0^{o}C) and left for 30 mins. Insoluble material was then collected by centrifugation (20 000 rpm, 20 mins, Beckman JA20 rotor, 0^{o}C) in glass tubes. The supernatants were dried by rotary evaporation, the residue being dissolved in SDS sample buffer. The pellets were split into two equal halves and one resuspended in 0.5 mls of ethanol, the other in 0.5 mls of water before each was added to 12.5 mls of ice cold chloroform:methanol, left slowly stirring for 14-16 hours, before centrifugation to remove insoluble material. The solutions were decanted through glass wool, rotary evaporated to dryness and both the dried solution and pellet resuspended in SDS-containing sample buffer. Fractions from each stage were electrophoresed on SDS-polyacrylamide gels, fixed, stained, destained, dried and auto-radiographed.

Figure 5.4. (a) and (b) Optimisation of lipid pre-extraction of the chromaffin granule membrane.

(a) and (b). 10-15% exponential gradient SDS-polyacrylamide gel of fractions during the series of two stage extractions of ^{14}C-DCCD labelled chromaffin granule membranes described in Chapter 5.2.4. TRACKS 1, chromaffin granule membranes; 3, acetone supernatant; 4, acetone:ethanol 3:1 supernatant; 5, acetone:ethanol 1:1 supernatant; 6, acetone ethanol 1:3 supernatant; 7, ethanol supernatant; 9-18 chloroform:methanol precipitates, odd tracks ethanol resuspended pellets, even tracks water resuspended pellets 9-10, acetone; 11-12, acetone:ethanol 3:1; 13-14, acetone:ethanol 1:1; 15-16, acetone:ethanol 1:3; 17-18, ethanol; 20-29, chloroform:methanol supernatants even numbered tracks, ethanol resuspended, odd numbered tracks water resuspended; 20-21, acetone prewash 22-23, acetone:ethanol 3:1 prewash; 24-25, acetone ethanol 1:1 prewash; 26-27, acetone:ethanol 1:3 prewash 28-29 ethanol prewash. TRACKS 2, 8, 19, 30 are blank.



(a) (b)



(c) (d)

Figure 5.4. (c) and (d) Optimisation of lipid pre-extraction of the chromaffin granule membrane.

(c) and (d) Autoradiographs of (a) and (b) respectively.

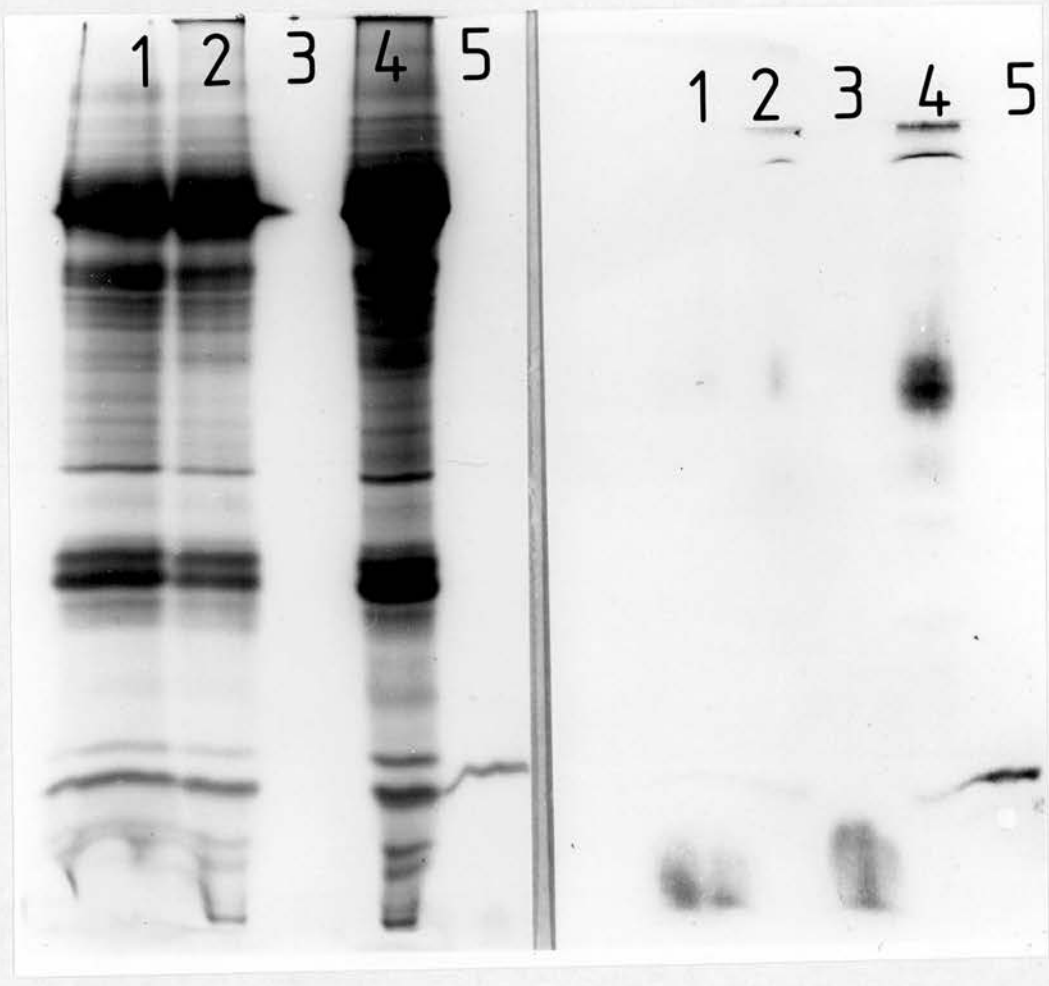


Figure 5.5. Two stage extraction of DCCD-reactive protein from chromaffin granule membranes

(a) 10-15% exponential gradient polyacrylamide gel of fractions from the purification of the DCCD-reactive protein from ^{14}C-DCCD labelled chromaffin granule membranes described in Chapter 5.2.4. TRACKS 1, chromaffin granule membranes; 2, precipitate from acetone:ethanol 1:1 pre-extraction; 3, acetone:ethanol solution; 4, chloroform:methanol 2:1 insoluble material; 5, chloroform:methanol solution.

(b) Autoradiograph of gel in (a).

The gels and autoradiographs are presented in Figure 5.4(a) to (d). It is quite clear that resuspension of the solvent washed membranes in ethanol results in almost complete insolubility of the DCCD-reactive protein in chloroform:methanol, and it is therefore important that the aqueous content of the solution be maintained. Tracks 20 and 21 clearly show that acetone washing results in relatively incomplete removal of the lipids, whilst autoradiography of tracks 11-18 show that a decreased fraction of the DCCD-reactive protein is extracted when ethanol is present in the pre-extraction medium. However since purity of the protein was desirable in all subsequent experiments acetone:ethanol 1:1 was used in the pre-extraction mixture. A gel and autoradiograph of chromaffin granule membranes extracted by this method is presented in Figure 5.5.

5.2.5 Further purification of the DCCD-reactive protein

It is obvious from the colour of the chloroform:methanol solution, which gradually turns brown on standing for some time at $4^{o}C$, that the extracted protein, although freed of other contaminants, as seen by SDS-gel electrophoresis, is associated with some non-protein material, of low molecular weight. In order to further purify the protein whilst keeping it in a soluble form a hydrophobic molecular exclusion column was chosen (Sephadex LH20). Chromatography by this method has been successfully used to separate brain proteolipids (Soto et al., 1969) from lipid contaminants using a step gradient of chloroform to chloroform:methanol. However, some difficulty was encountered when trying to resuspend rotary evaporated material in organic solvent before column chromatography, even if large amounts (up to 20 volumes) of solvent were used. This could have been due to aggregation of the rotary evaporated proten. In consequence a different method of rotary evaporation was used, which although based upon the method described

by Fillingame (1976), varies sufficiently from that method to warrant a further description here. Chloroform:methanol extracts could be stored apparently without deterioration for long periods at 0°C (up to one month at least). The water-bath of the rotary evaporator was kept at 25°C. Raising this temperature above 40°C caused insolubility of the rotary evaporated material, although lowering of the water-bath temperature to 10°C had no noticeable effect. The material was rotary evaporated without bubbling by rotating the flask very fast, and not having it more than 40% full at any one time. The solution eventually turned cloudy upon rotary evaporation, and at this time the vacuum was released, the solution removed from the water-bath and chloroform: methanol added until the solution cleared. This declouding process was repeated until the solution reached a volume suitable for loading onto the column (up to 20 mls). If the solution turned cloudy after this time it could be clarified by the addition of 4% water.

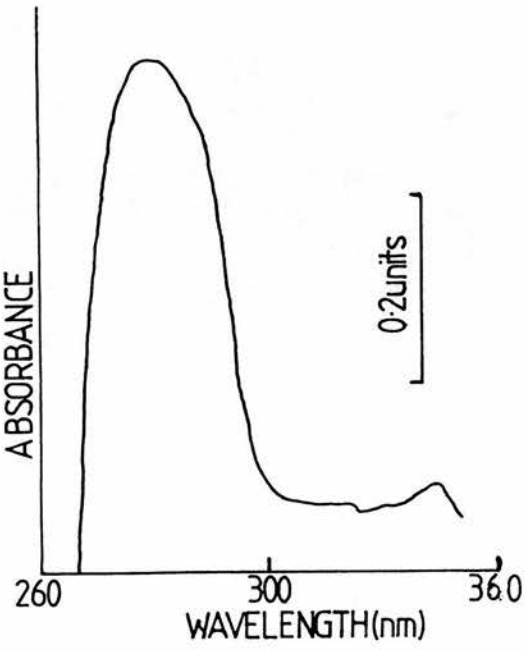
Sephadex LH20 chromatography. The sephadex LH20 gel slurry was prepared and the column poured as described by Soto et al. (1969). After initial problems with homemade columns a Pharmacia SR25/45 (Bed volume 30 cm x 5.1 cm^2) was purchased. Previous authors used a stepped gradient (Cattell et al., 1971) from chloroform (in which the slurry floats) to chloroform:methanol in which it sinks. As the volume of the slurry and the relative solution/slurry density change while the column is running, it was found essential to weight the top of the column with sand before use. The multistep gradient previously used required manual changing of solutions at each stage, previous authors using up to six changes of solution during chromatography. This was both laborious and time consuming and since contamination from other proteins was negligible, was not essential. A linear chloroform to chloroform:methanol 1:1 gradient was therefore used. The total volume

of the gradient was made equivalent to the total volume of the step gradients previously used for the 150 ml column, divided equally between chloroform (250 mls in the mixing chamber) and chloroform: methanol, 1:1 (250 mls in the supply chamber). To prevent retrograde flow the mixing chamber was 5 cm below the supply chamber. The column was run under gravitational pressure.

The simplest method of loading protein onto the gel matrix before chromatography was by removal of the top of the column and pipetting the protein onto the sand (the column was equilibrated in chloroform and sample loaded in chloroform:methanol) allowing the material to run into the gel matrix before affixing the column topand running the gradient. If the column stopped it could be restarted without any noticeable effect. If the column dried out it could be restarted by use of de-gassed chloroform:methanol until the bed repacked (and protein successfully eluted). Fractions of 7.5 mls were collected in tubes which had been washed with persulphuric acid to remove any organic contaminants. Batches of five fractions (total volume 37.5 ml) were rotary evaporated to dryness and resuspended in 2 mls of chloroform: methanol with 80 µl of double distilled water. At this stage samples could be stored for long periods of time. However, the samples gradually turned yellow if left for more than three weeks, which suggested that some contaminants still remained.

5.2.6 Water solubility of contaminants

One of the 2 ml fractions from the column had accidentally had 160 µl of water added to it and this separated out forming the top layer, into which most of the yellow appeared to partition. Thus, a further purification step was introduced. This consisted of taking the 2 ml fractions which contained protein (see later) and gently



(a)

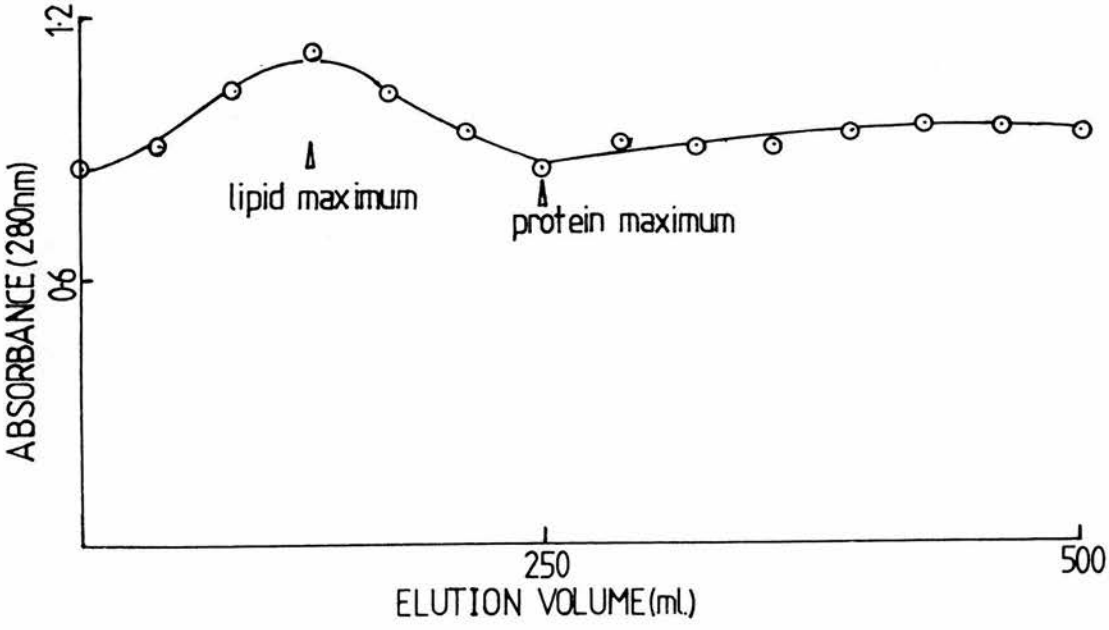


Figure 5.6. Detection of protein in chloroform:methanol solution

(a) Wavelength scan of protein rich peak from the LH20 column.

(b) Plot of absorbance at 280 nm of successive fraction from pooled concentrated samples from the LH20 column.

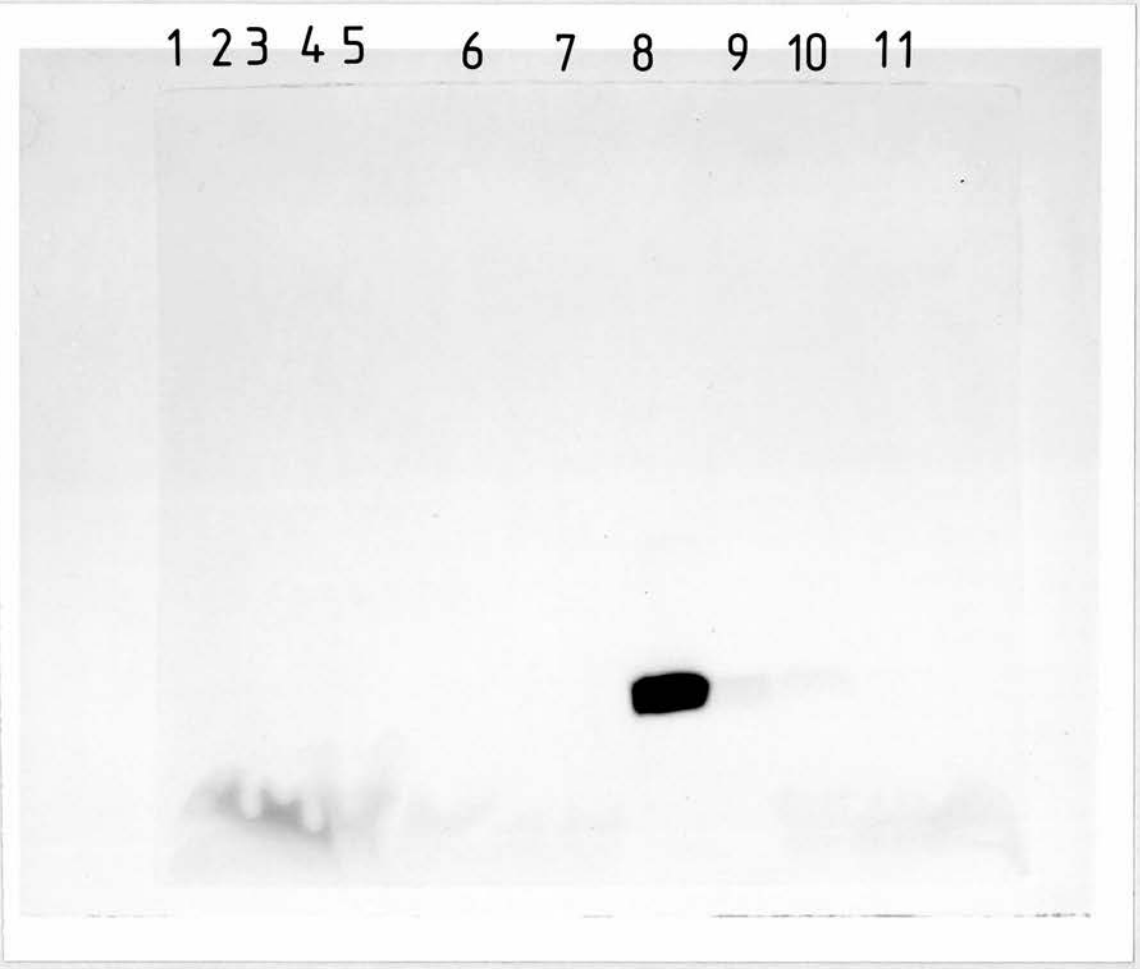


Figure 5.7. Elution of DCCD-reactive protein from Sephadex LH20 chromatography

10-15% exponential gradient polyacrylamide gel of successive fraction from LH20 chromatography of chromaffin granule DCCD-reactive protein from fraction 1 (chloroform) to fraction 12 (chloroform: methanol 1:1) as described in Chapter 5.2.5.

shaking with 0.25 mls of water, three times, each time removing the top layer. SDS gel electrophoresis indicated that about 10% of the total protein partitioned into the water-soluble phase. The remaining chloroform solution was a pale yellow in colour and whilst the protein was a pale brown when the solution was dried neither further water or acetone washing steps removed any of the colour. This indicated that the remaining contaminant was either insoluble in both these solvents, or was very firmly bound to the protein.

5.2.7 Detection of the protein

Absorbance measurements at 280 nm on the column fraction from the column (Fig. 5.6(b)) showed no observable increase in the fractions which contained the protein, probably due to the high background absorbance at 260 nm (Fig. 5.6(a)) and low protein concentration, and this therefore could not be used to locate the extracted protein in the column eluates. To investigate the possibility of using gel electrophoresis to visualise the protein, 100 µl samples of the column fractions were dried down in a stream of N_2, redissolved in SDS sample buffer and subjected to electrophoresis on SDS polyacrylamide gels (10-15% exponential gradient) as described previously. After staining and destaining the protein-containing fractions could be identified visually. A typical gel can be seen in Figure 5.7. Tracks 2-4 (containing column-fractions 11-20) contained most of the brown coloured material whilst tracks 6-8 contained most of the Coomassie blue staining protein band. Thus the protein was eluted from the column by chloroform:methanol in the ratio 8:1-4:1. Some high molecular weight material appears to elute just before the DCCD-reactive protein but it does not contaminate the final product.

SAMPLE	Protein µg/ml	Volume (ml)	Total protein	Relative protein %
Starting membranes	8.6×10^3	24	2.06×10^3	100
Acetone/ethanol supernatant	78	5	3.9	0.165
Acetone/ethanol precipitate	7.86×10^3	25	1.97×10^3	95.63
Chloroform/methanol supernatant	21	10	2.1	0.102
Chloroform/methanol precipitate	3.92×10^4	5	1.96×10^3	95.15
LH20 chromatography fractions 2-4	2.4	6	0.14	0.0068
fractions 6-8	31.5	6	1.89	0.092

Table 5.4. Distribution of protein during purification of chromaffin granule DCCD-reactive protein

The table shows the total amount of protein in successive fractions during a typical purification procedure as described in Chapter 5.3.1.

5.3.1 Assessment of the content of DCCD-reactive protein in chromaffin granule membranes

It is of interest to estimate the concentration of this protein in chromaffin-granule membranes, as a fraction of the total membrane protein; and to compare this with figures derived from experiments on isolated F_1-like ATPase (Apps and Glover, 1978; Apps and Schatz,1979). Further, estimates as to the number of polypeptides per granule could be made (see Winkler and Westhead, 1980).

Method. 24 mls of chromaffin granule membranes (8.6 mg protein. ml^{-1}) were extracted with 600 mls of acetone:ethanol 1:1. After five minutes at $4^{o}C$ the solution was centrifuged to precipitate insoluble material. The supernatant was rotary evaporated to dryness and the residue redissolved in 1% SDS 0.5 M NaOH. The pellet was resuspended in 25 mls of distilled water, and a sample (1 ml) kept for protein estimation. The resuspended pellet was added, to 24 volumes of ice cold chloroform:methanol and left sitrring for 5-6 hours. The solution was then centrifuged to remove insoluble material, the supernatant carefully decanted through a glass wool filter, and the pellet resuspended in alkaline SDS solution as before. The chloroform:methanol extract was then rotary evporated, split into two, half dried and redissolved in alkaline SDS solution, whilst the other half applied to Sephadex LH20 and chromatographed as described in 5.2.5. Fractions 2-4 and 6-8 were dried down and redissolved in alkaline SDS solution. Protein estimations were carried out by the modified Lowry assay described in Chapter 2.

Results of this experiment are presented in Table 5.4. The purified DCCD-reactive protein was calculated to comprise 0.092% of the total membrane protein, although this may well be an underestimate of the total amount, since in Figure 5.5(b), tracks 4 and 5 clearly show that not all of the DCCD-reactive protein is extracted into chloroform:

methanol.

In Chapter 3.2 were reported experiments in which ^{14}C-DCCD labelled chromaffin granule membranes were dissolved in SDS, subjected to electrophoresis and a radioactivity profile obtained by slicing a track from the gel. Since the amount of membrane protein loaded onto the track was exactly 100 µg an estimate of the covalent binding of ^{14}C to protein was obtainable. The total radioactivity in the peak was 3734 dpm. The specific radioactivity of the ^{14}C-DCCD used was 50 Ci. mol^{-1}, which gives an estimate of 340 pmoles DCCD reacting per mg protein. This Figure is 2.6 times larger than the amount derived from the estimates of purified protein (M_r = 7600) which is 130 pmoles mg^{-1} membrane protein.

5.3.2 Assessment of ^{14}C-DCCD:DCCD-reactive protein stoichiometry

In order to be able to assess the fraction of the DCCD-reactive protein which extracted into chloroform:methanol, as well as obtaining an estimate of the relative amounts of bound DCCD to DCCD-reactive protein a similar experiment to 3.2 was performed.

Method. Chromaffin granule membranes 6.8 mg protein ml^{-1} were labelled with ^{14}C-DCCD in the presence of 10 mM ATP (16 h, 4^{o}C) and extracted into chloroform:methanol as described in 5.2.4, using 4 mls of membrane suspension. At this stage 50 mls of the chloroform:methanol extract was dried down and redissolved in SDS-containing sample buffer (1 ml). The other 50 mls were concentrated to 5 mls in chloroform: methanol and ^{14}C-DCCD (10 µl 2 mM, specific radioactivity 50 Ci.mol^{-1}) added. This mixture which shall be referred to as the post-labelled extract, was left for 12-16 h (0^{o}C). After drying down in a stream of N_2, the postlabelled extract was redissolved in 1 ml of SDS-containing sample buffer. This, with the other sample (known as the once-labelled

extract) were subjected to SDS-gel electrophoresis. The gel was fixed, stained, destained, tracks containing 150 µl of both once and postlabelled extract excised and radioactivity estimated as described in Chapter 2.

Incorporation of radioactivity in the once-labelled extract was 3304 dpm, and in the postlabelled extract 8920 dpm. Calculations analogous to those above give a value of 148 pmoles/mg protein for the once labelled extract, and 400 pmoles DCCD/mg protein for the post-labelled extract. Comparison of ^{14}C incorporation into the once-labelled extract with the amount of protein obtained from protein purification, which is 130 pmoles/mg protein, gives a DCCD:DCCD reactive protein stoichiometry of approximately 1:1. The same figure for the post-labelled extract is 2.8:1. The result for the once labelled extract agrees with the estimates obtained for the stoichiometry of DCCD:DCCD-reactive protein of chloroplasts and N. crassa (Sigrist-Nelson et al., 1978; Sebald et al., 1979). Other workers have reported a value of 0.17 nmoles DCCD/nmol DCCD reactive protein as being sufficient to inhibit maximally the ATPase activity (Fillingame, 1976; Sigrist-Nelson et al., 1978). These authors did not study the effect of DCCD at higher concentrations than the minimum required to give maximal inhibition of the ATPase activity and it may well be that at a higher concentration of DCCD a 1:1 stoichiometry can be obtained.

The postlabelled extract, has approximately three DCCD molecules bound to each polypeptide chain. If it is assumed that the DCCD-reactive protein is completely denatured in chloroform:methanol (the medium in which the second labelling takes place), that the protein is modified by DCCD only on carboxyl residues when redissolved in SDS, and that the carboxyl terminus of the protein is unblocked, then the number of free carboxyl residue can be estimated. This value is three per

protein. One is possibly the C-terminus, but the other two must be due to carboxyl amino acid side chains. This gives an estimate of the ratio of asparagine + glutamine:aspartate + glutamate derived from the amino acid analyses. However several assumptions have been made in deriving this ratio and so this result must be treated with caution.

The estimate of the amount of DCCD-reactive protein obtained from slicing of labelled chromaffin granule membranes (discussed in 5.3.1) of 340 pmoles/mg protein, coupled with the derived stoichiometry of DCCD:protein, the observation that labelled band remains in the chloroform:methanol precipitate and the value of 148 pmoles/mg protein for the extract gives a value of only 38% of the DCCD-reactive protein being extracted with chloroform:methanol. A more accurate estimate of the amount of DCCD-reactive protein in the chromaffin granule membrane is therefore about 300-350 pmoles/mg protein.

Finally results obtained from amino acid analyses of the purified protein suggest that the protein estimations are slightly inaccurate, and that a better estimate of DCCD-reactive protein is closer to 240-300 pmoles/mg protein. How does this compare with the amount of F_1-ATPase in the chromaffin granule membrane?

5.3.3 Estimation of F_1ATPase content by immune replica

Because no figures have been published for the amount of F_1-ATPase purified from chromaffin granule membranes (Apps and Glover, 1978; Apps and Schatz,1979), previous authors were forced to calculate the content of F_1ATPase based upon activity measurements (Winkler and Westhead, 1980). Since both the specific activity and pH optimum of the F_1ATPase changes when detached from the membrane, the value of 1.4% total protein derived by these authors is at best a rough estimate of the ATPase content. A more accurate estimate may be obtained from

Fraction	c.p.s./μg
F_1ATPase	235
Adrenal mitochondria	24
Chromaffin granule membranes	0.74

Table 5.5. Protein A binding to anti beef heart F_1ATPase antibodies of various samples.

Protein which had been labelled with ^{125}I was detected by γ counting of F_1 fractions excised from a cellulose nitrate replica of a 10%-15% exponential gradient polyacrylamide gel. Methods as described in the text.

quantitation of the binding of anti-F_1 globulins to chromaffin granule membranes, using the immune replica technique (Towbin et al., 1979) and "decoration" of the immune complex with ^{125}I-protein A. Comparison of the number of counts for purified F_1ATPase, compared to either chromaffin granule membranes or adrenal mitochondria, will give a reasonable estimate of the F_1ATPase content of these membranes.

Method. F_1ATPase was purified by chloroform extraction of purified adrenal mitochondria (Beechey et al., 1975). Adrenal mitochondria and chromaffin granule membranes were purified as described in Chapter 2. The protein content of these fractions were measured by the modified Lowry method, on acetone:ethanol washed material, as described in Chapter 2. Several different concentrations of purified F_1ATPase and adrenal mitochondria were loaded onto tracks of a 10% SDS polyacrylamide gel. A second gel contained several different preparations of chromaffin granule membranes and adrenal mitochondria. The gels were electrophoresed, protein transferred to cellulose nitrate sheets and immune replicas made as described in Chapter 2 using anti F_1-ATPase antiserum. The cellulose nitrate sheets were dried, autoradiographed and the part of the sheet containing anti F_1ATPase excised and counted for radioactivity in a Wilj gamma counter.

The results of this experiment are presented in Table 5.5. From the data presented in Table 5.5, the F_1 content of adrenal mitochondria was calculated to be 10.3% of the total membrane protein. The figures also indicate that chromaffin granule membranes have an F_1 content which is 3.1% that of adrenal mitochondria electrophoresed on the same gel. Figures obtained using anticytochrome oxidase antibodies, in an analogous experiment, give a figure of 1.4% contamination of chromaffin granule membranes by mitochondria, leaving the F_1 content in chromaffin granules as 1.7% of the mitochondrial level or 0.17% of the total

membrane protein. However careful examination of Figure 3(B) from Apps and Schatz (1979) shows that in quantitative microcomplement fixation experiments, the chromaffin granule ATPase reacts more weakly against antibodies raised against beef heart mitochondrial F_1-ATPase than the ATPase from beef heart mitochondria. From the 50% fixation value a factor of 2.1 can be derived. In these experiments a different batch of antiserum was used, and the titre of the antibody used here may vary from that of Apps and Schatz (1979). The relative reactivity of the chromaffin granule ATPase is likely to be of the same order and so a factor of 2.1 is used. This gives a value of 0.36% of the total chromaffin granule membrane protein as F_1ATPase. If an Mr of 360 000 is assumed for the F_1 (consistent with a subunit stoichiometry of $\alpha_3\beta_3\gamma\ \delta\ \varepsilon$) there are 10 pmoles ATPase/mg membrane protein. Taking a value of 240-300 pmoles/mg protein for the amount of DCCD-reactive protein, the stoichiometry of DCCD-reactive protein: F_1ATPase is 24-30. Calculations based upon the lipid content of the chromaffin granule membrane analogous to those performed by Winkler and Westhead (1980) give a figure of 5 ATPase molecules and 96-128 molecules of DCCD-reactive protein per granule.

Although the stoichiometry of DCCD-reactive protein: F_1ATPase is relatively high several factors remain undetermined which may account for the observed data. Firstly the chromaffin granule F_1ATPase may not transfer as well as mitochondrial F_1ATPase onto nitrocellulose sheets. Secondly some F_1ATPase may be lost from that granule by the preparation procedure. One observation that favours such an explanation is the DCCD induced enhancement of ATP stimulated catecholamine uptake (Apps et al., 1980b) in that DCCD is blocking protonpores left by removal of F_1ATPase. In fact only 50-75% of the ATPase needs to be lost to bring the stoichiometry into reasonable figures; that is a ratio of

6-12 DCCD-reactive proteins per ATPase. Thirdly, many other assumptions have been made in deriving the data, which may lead to uncertainties in the calculations.

5.4 Summary

1. Chromaffin granule DCCD-reactive protein has been purified from highly purified chromaffin granule membranes in four stages.

2. These stages were (a) delipidation of the membranes by acetone: ethanol 1:1; (b) extraction into chloroform:methanol 2:1; (4) column chromatography in organic solvents using Sephadex LH20 and (d) washing of column eluate with water and acetone.

3. Calculation based upon the extraction procedure and ^{14}C-DCCD labelling suggest (a) the DCCD:protein stoichiometry of proteins labelled in situ is 1:1 (b) there are two free carboxyl amino acid side chains on the protein and (c) that only 38% of the protein is extracted into chloroform:methanol.

4. The amount of DCCD reactive protein is 240-300 pmoles/mg membrane protein which gives a figure of 96-128 copies of the protein per granule. The F_1ATPase is at a concentration of 12 pmoles/mg membrane protein, giving 5 copies per granule and an F_1ATPase:DCCD reactive protein stoichiometry of 1:24-30.

Chapter 6

Characterisation of chromaffin granule membrane DCCD-reactive protein

6.1 Introduction

Since a discussion of the sequences of DCCD-reactive proteins from various sources has been made in Chapter 1, the introduction to this chapter will be limited to consideration of amino acid analyses and N-terminal determinations. Some of these results are based on sequence analyses of DCCD-reactive proteins. The earliest work on characterisation of the chloroform:methanol-soluble protein was done on immune precipitated ATP-ase complex using antibodies raised against yeast mitochondrial F_1ATPase (Sierra and Tzagaloff, 1973). Analysis of the protein from *E. coli* has been reported but subsequent work, based upon sequence analysis (Sebald et al. 1979(b), Altendorf et al. 1979) has revealed minor differences from the earlier results. The amino acid content of DCCD-reactive protein from the plasma membrane of the thermophilic bacterium PSIII has also been reported (Sone et al. 1978) but those results differ from those derived from the sequence analysis (Sebald et al. 1979(b)). Amino acid analysis of the butanol-extracted lettuce chloroplast DCCD-reactive protein has been reported (Sigrist-Nelson and Azzi, 1979) and differs markedly from the results of Sebald et al. 1979(b). This difference could be due either to a species difference or to the presence of two different DCCD-reactive proteins in the chloroplast membrane. Analysis of the DCCD-reactive proteins from mitochondria of beef heart and *N. crassa* has also been published (Sebald et al. 1979(a) and (b)).

Organic solvent soluble proteins have also been isolated from other ion translocating ATPases, and an analysis of these was reported as early as 1972 (Maclennan et al.) for the sarcoplasmic reticulum Ca^{2+} ATPase. More recently Knowles et al. (1980) suggested that there are at least four distinct organic solvent soluble polypeptides isolable from sarcoplasmic reticulum, although their precise function has not yet

	Mitochondria				Chloroplast		Plasma Membrane				Lamb Kidney		SR Ca^{2+} ATPase
Source of PL	Yeast		*N.crassa*	beef heart	Lettuce	Spinach	*E. coli*		PSIII		Na^+/K^+ ATPase		
Reference													
Amino acid	(a)	(b)	(b)	(c)	(d)	(e)	(e)	(f)&(c)	(a)	(c)	(h)I	II	(i)
Asx	3	3	4	3	4	2	5	5	1	1	10	6	5
Thr	3	3	2	3	3	3	1	1	3	3	3	5	7
Ser	4	5	5	5	5	3	0	0	3	3	5	7	8
Glx	2	2	5	3	6	7	4	4	5	5	10	8	10
Pro	2	2	1	1	5	4	3	3	3	3	5	4	2
Gly	8	10	11	11	10	11	10	10	10	11	9	7	3
Ala	8	10	14	13	12	17	14	13	9	9	8	10	2
Cys	n.d.	1	0	1	0	0	0	0	0	0	2	2	1
Val	5	6	6	4	6	7	6	6	7	8	5	6	6
Met	2	3	4	3	1	2	8	8	2	2	1	2	2
Ile	7	9	6	7	5	6	8	8	7	9	6	6	4
Leu	10	12	11	9	10	12	13	12	8	10	9	12	17
Tyr	1	1	2	2	1	1	2	2	1	1	5	3	7
Phe	6	6	6	7	4	3	4	4	3	3	5	6	5
His	n.d.	0	0	0	0	0	0	0	0	0	2	2	1
Trp	n.d.	0	0	0	n.d.	0	0	0	n.d.	0	n.d.	n.d.	n.d.
Arg	1	1	2	1	2	2	2	2	4	4	6	4	10
Lys	2	2	2	2	2	1	1	1	0	0	7	4	1
Total No.	64	75	81	75	76	82	81	80	68	73	102	94	94

Table 6.1. Amino acid analyses of DCCD-reactive proteins and proteolipids from ion translocating ATPases, based upon data from several sources. (a) Sierra and Tzagaloff, 1973; (b) Sebald et al. 1979a; (c) Sebald et al. 1979b; (d) Sigrist Nelson et al. 1979; (e) Fillingame, 1976; (f) Altendorf et al. 1979; (g) Sone et al. 1978; (h) Reeves et al. 1980; (i) Maclennan et al. 1972. Values given are the best integral value of individual residues.

been discussed and the polypeptides have not been purified to homogeneity. Two distinct chloroform:methanol soluble proteins have been isolated from lamb kidney plasma membrane Na^+/K^+ATPase (Dowd et al. 1976). A compilation of some of these results appears in Table 6.1.

From this table several points emerge. There is a total absence of histidine and tryptophan from all the DCCD-reactive proteins isolated to date, and a lack of cysteine in several of them also makes these proteins unusual. In contrast, the chloroform:methanol soluble proteins so far isolated from Ca^{2+}- and Na^+/K^+-ATPases contain both cysteine and histidine (tryptophan not having yet been determined). The high content of the hydrophobic amino acids glycine, alanine, leucine, isoleucine, valine and phenylalanine in all the organic solvent-soluble proteins is probably the factor which accounts for their unusual solvent solubility properties.

The identity of the N-terminal amino acid of the DCCD-reactive proteins depends to some extent on the site of synthesis. Those proteins synthesised within the mitochondria (such as occurs in S. cerevisiae) invariably have N-formyl-methionine as the N-terminus. On the other hand, the N-terminal of those mitochondrial proteins in which the site of biosynthesis has been shown to be on cytoplasmic, rather than intra-mitochondrial ribosomes (From N. crassa, beef heart and mouse liver mitochondria), the N-terminus is unblocked and variable (Sebald et al. 1979(a), Aroskar and Avadhani, 1980). In prokaryotes the N-terminus of all DCCD-reactive proteins so far isolated has been shown to be N-formyl-methionine.

6.2.1 N-terminal determination

One of the standard criteria for homogeneity of a purified protein is the demonstration of a single N-terminal amino acid in the preparation.

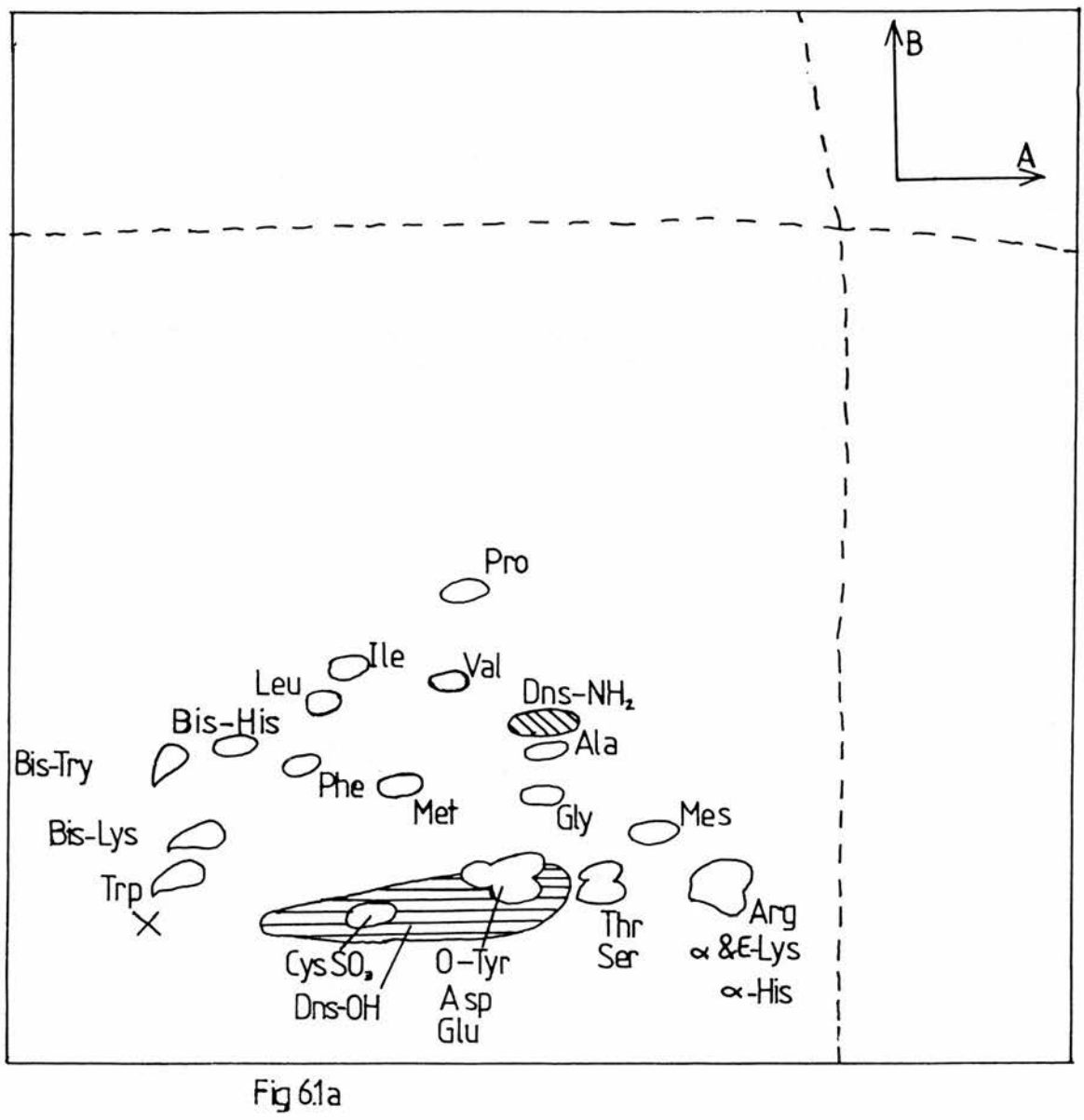


Fig 6.1a

Figure 6.1. Migration of DNS amino acids on polyamide plates

(a) Conditions are as described in the text and figure (X 4) shows the pattern obtained after two dimensions.

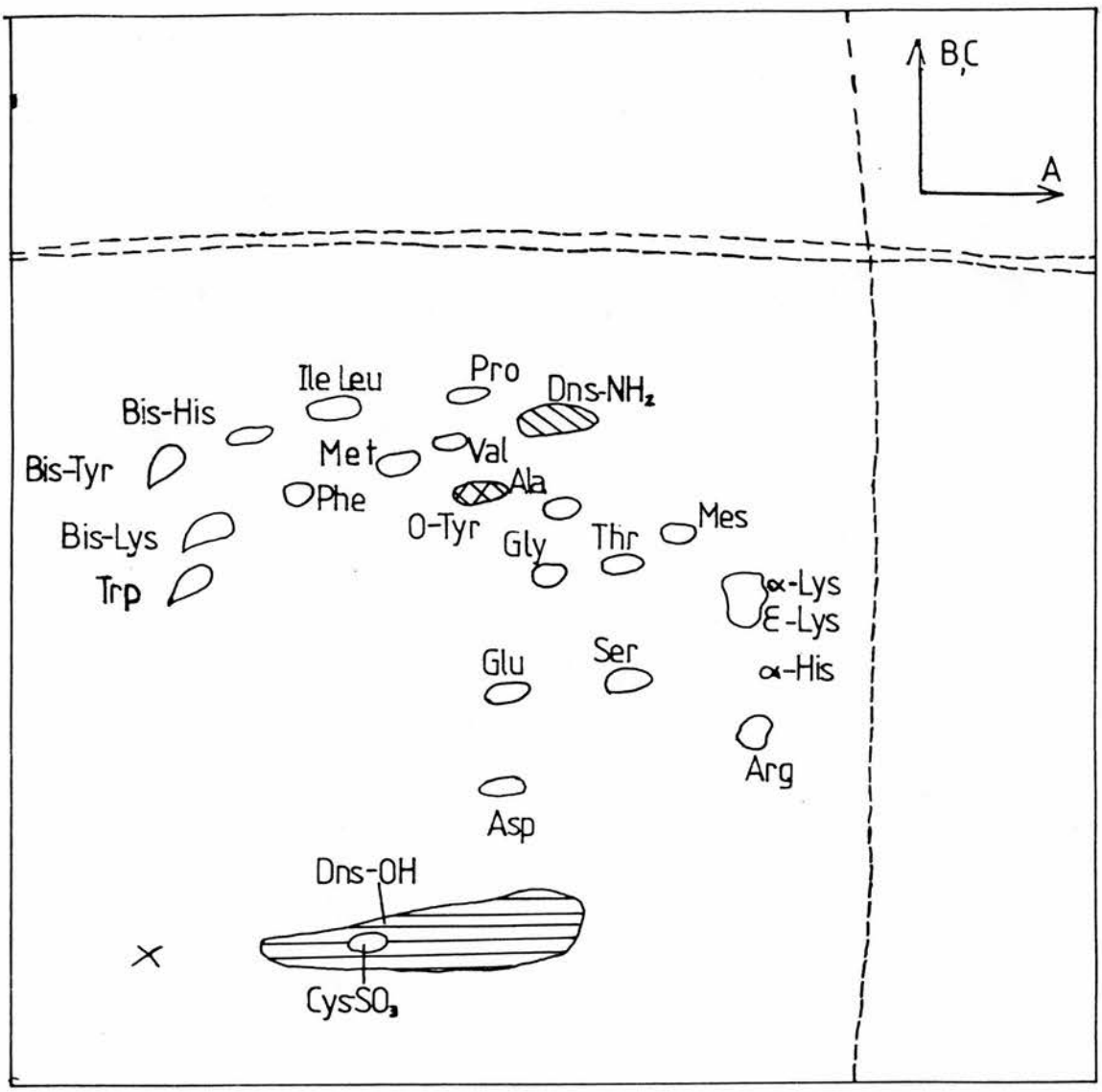


Fig 6.1b

Figure 6.1. Migration of DNS amino acids on polyamide plates

(b) Migration of DNS amino acids on polyamide plates. Conditions are as described in the text and figure (X 4) shows the pattern obtained after three dimensions.

For the purpose of the work done in this thesis, it was a very important consideration, as the DCCD-reactive protein from bovine adrenal mitochondria could have been a contaminant of the purified chromaffin granule protein (although gel electrophoresis of the purified protein suggested that it was not). If the adrenal mitochondrial DCCD-reactive protein were the same as that from beef heart (as is likely to be the case), then the N-terminus would be aspartate (Sebald et al. 1979(a)) and would be detected by the DNS-chloride procedure described below.

Method. As a control experiment 10 nmoles of sperm whale myoglobin was dissolved in SDS and labelled with DNS-Cl as described in Chapter 2. The labelled protein was precipitated with acetone and hydrolysed in 6 M HCl for 20 h at 105^{o}C in a tube sealed under atmospheric pressure. The hydrolysate was vacuum desiccated over NaOH pellets and the solid material resuspended in 50% aqueous pyridine prior to thin layer chromatography on polyamide plates as described in Chapter 2. Figure 6.1(a) and (b) shows the separation of standard DNS amino acids after two and three dimensions respectively. The main point to note in the standards are that after two dimensions leucine and isoleucine can be differentiated but not after three dimensions have been chromatographed. Figure 6.2(a) and (b) shows the migration of DNS amino acids derived from DNS-Cl-labelled, acid hydrolysed sperm whale myoglobin after two and three dimensions of chromatography. This shows the presence of DNS-e-lysine, DNS-o-tyrosine, DNS-OH, DNS-NH_2, DNS-valine and another spot which migrated in a similar manner to leucine but slightly ahead of this amino acid in the third dimension. There is also a minor spot of glycine which is probably due to contamination of the sample. The other spot could be identified as a peptide (Valentine, Ph.D. thesis, 1978), the dansyl derivative of valyl-leucine. This result suggests that the N-terminal sequence is

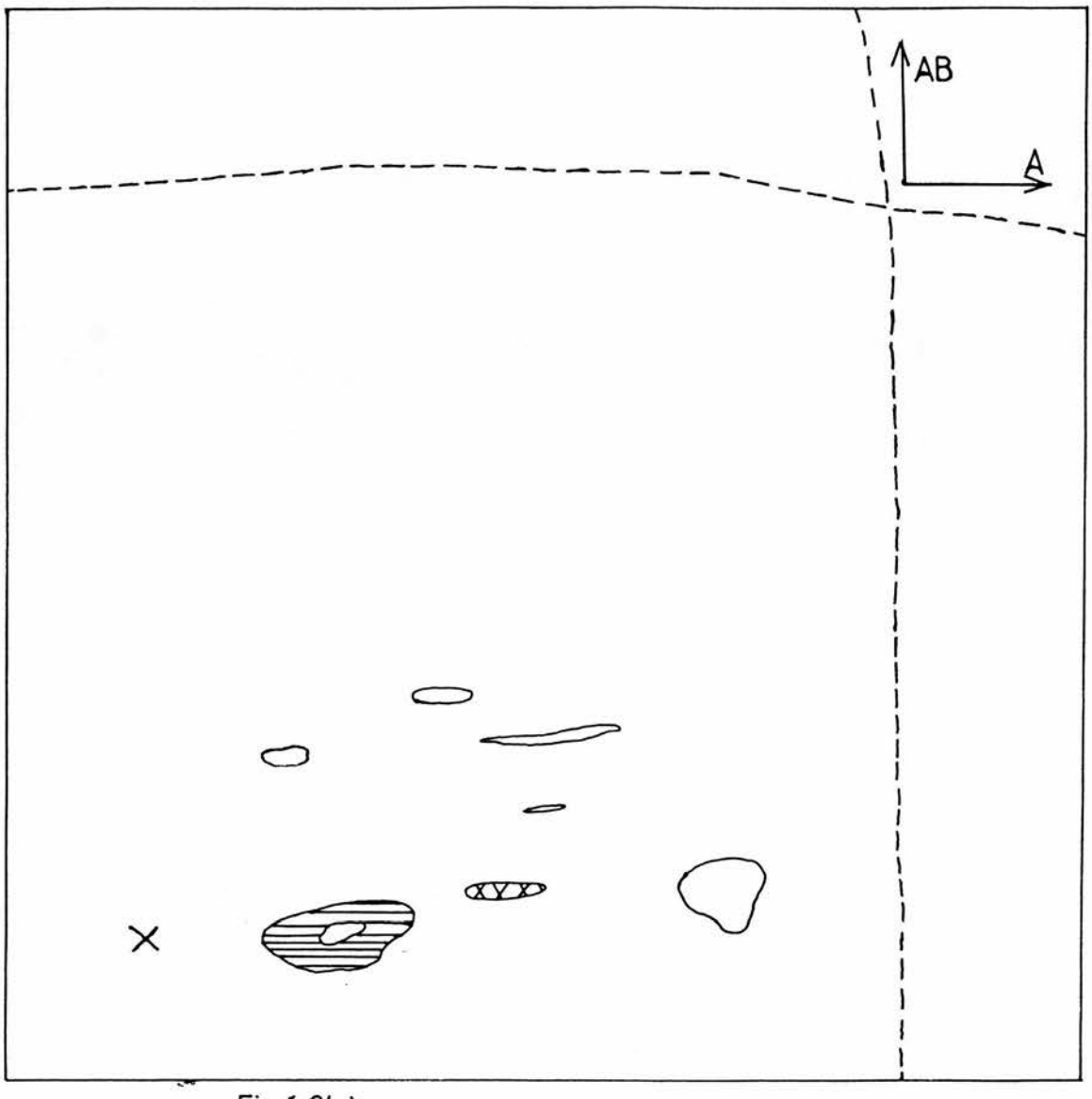


Fig. 6. 2(a)

Figure 6.2. N-terminal amino acid determination of sperm whale myoglobin.

Myoglobin was dansylated and hydrolysed as described in the text. The hydrolysate subjected to chromatography.

(a) Pattern obtained after two dimensions (X 4).

(b) (overleaf) Pattern obtained after three dimensions (X 4).

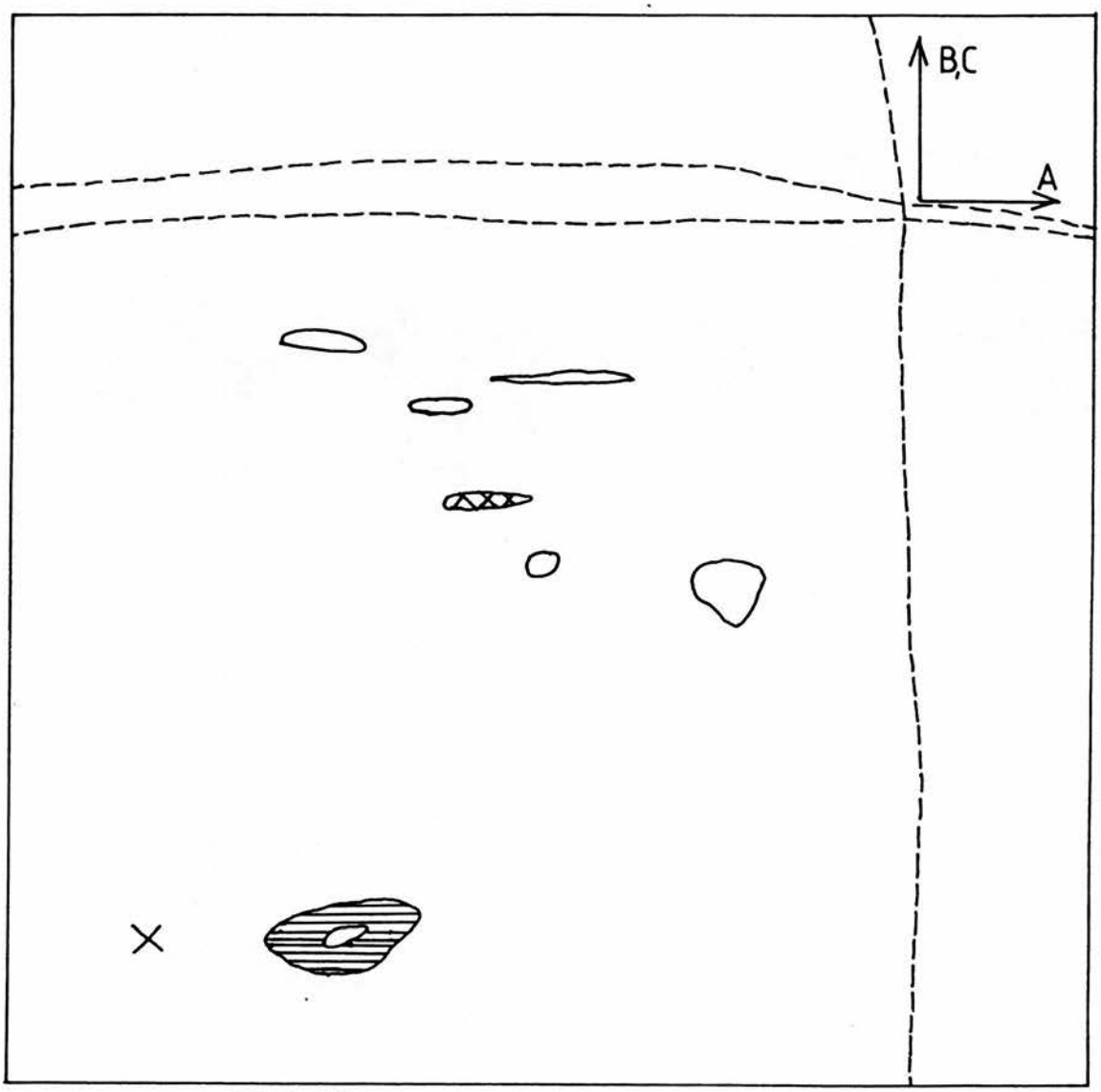


Fig 6.2(b)

probably N-valine-leucine and that the dipeptide is found because of the difficulty of hydrolysing isoleucine, leucine, and valine peptide bonds which has been ascribed to steric hindrance (Glazer et al. 1975). When checked against sequence analysis of sperm whale myoglobin the results obtained here agreed closely with the sequence determination (Edmundson, 1965).

6.2.2. Dansylation of the DCCD-reactive protein

Having presented evidence that the modification of the dansylation procedure (Gray, 1972) for N-terminal amino acid determination is successful in labelling the N-terminus, an attempt to identify the N-terminus of the chromaffin granule DCCD-reactive protein was made.

Method. Approximately 10 nmoles of purified chromaffin granule DCCD-reactive protein (purified as described in Chapter 5) was dried down from the chloroform:methanol solution, in which it was stored, in a gentle stream of nitrogen gas. The protein which was dissolved in 100 µl 1% SDS appeared to form an opaque colloidal solution in SDS. To this, 100 µl of N-ethylmorpholine was added. This did not appear to affect the solubility of the protein. Then 100 µl of freshly dissolved DNS-Cl was added (25 mg ml^{-1} in dimethyl formamide). The protein appeared to slowly 'float' to the top of the solution, and so the solution was gently vortexed every five minutes for the time of incubation. After one hour, the protein was precipitated with 1 ml of acetone ($0^{o}C$) and the mixture washed three times by resuspension and centrifugation in acetone. The final pellet fluoresced bright green under ultraviolet light. 0.3 mls of 6 M HCl was added to the protein, the tube sealed under atmospheric pressure and incubated for 20 hours ($105^{o}C$). The acid-hydrolysed protein was then vacuum desiccated over NaOH pellets and the contents redissolved in 50% aqueous pyridine.

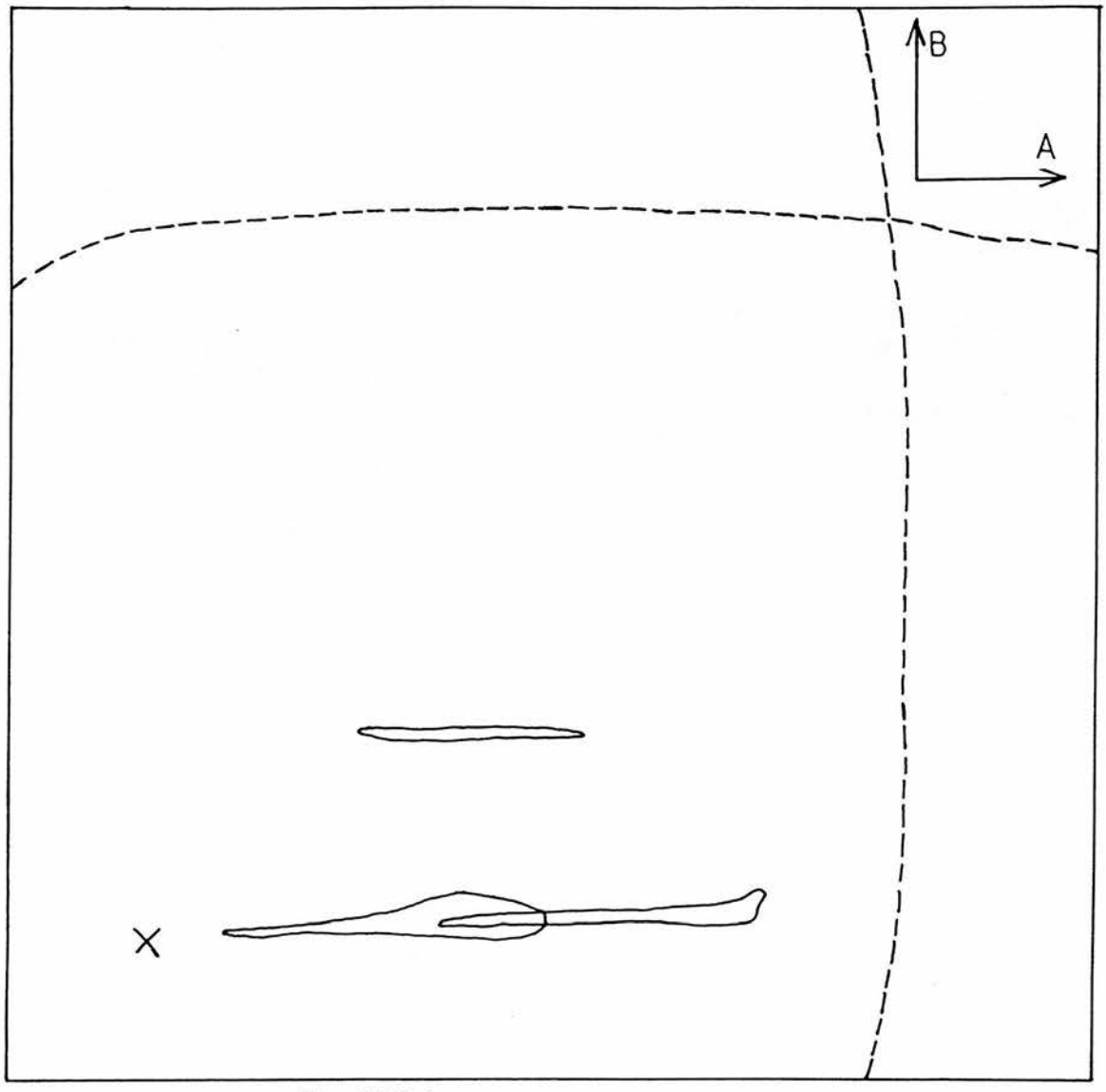


Fig 6.3(a)

Figure 6.3. N-terminal amino acid determination of chromaffin granule DCCD-reactive protein

Chromaffin granule DCCD-reactive protein was dansylated and hydrolysed as described in the text. The hydrolysate was then subjected to chromatography on polyamide gels as described in Chapter 2.

(a) Pattern obtained after two dimensions (X 4)

(b) (overleaf) Pattern obtained after three dimensions (X 4).

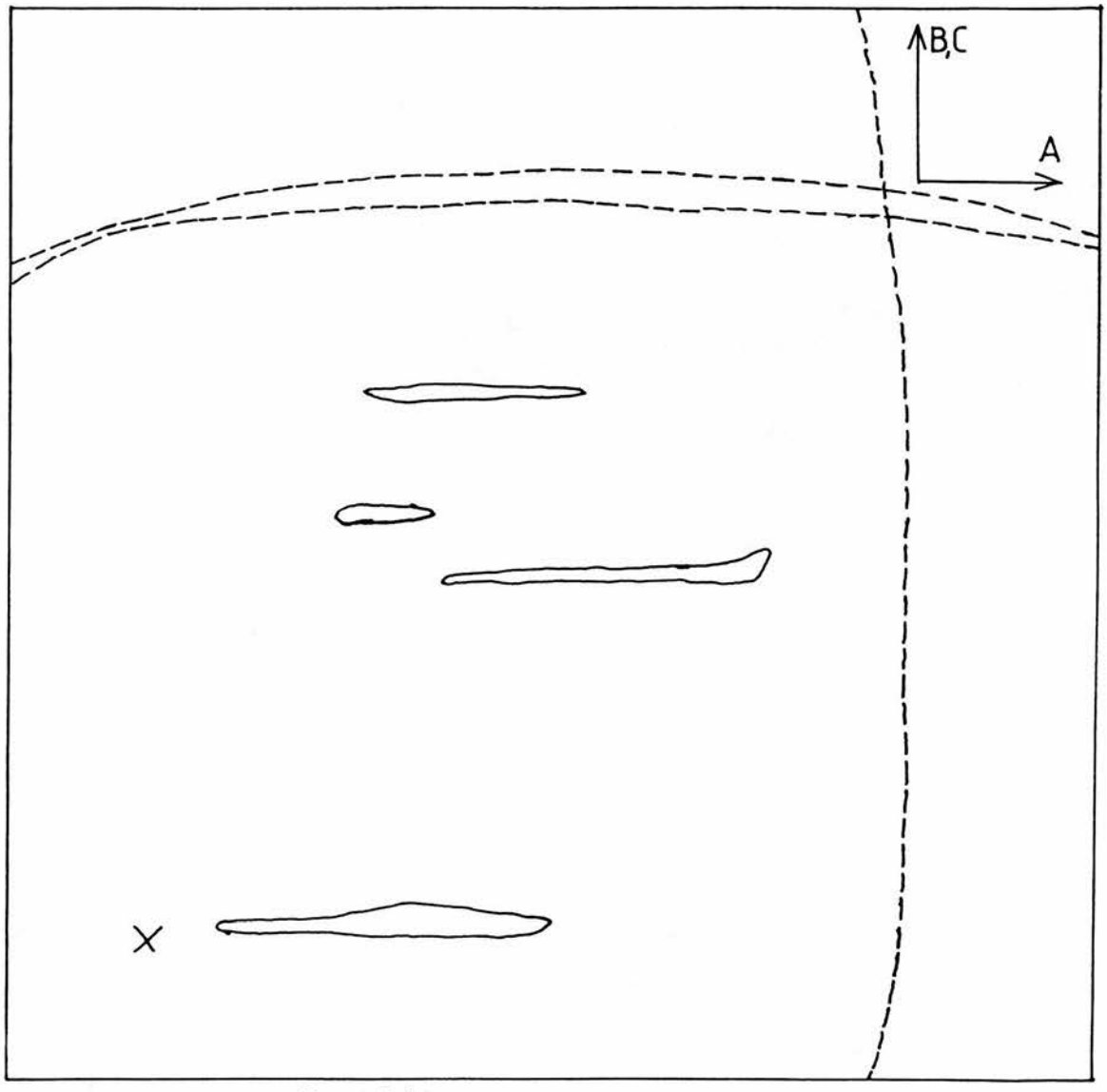


Fig 6.3(b)

Approximately 1 nmol of the DNS-Cl treated, acid hydrolysed protein in 50% aqueous pyridine, was then subjected to three dimensional thin layer chromatography as described in Chapter 2.

The results of the chromatography after two and three dimensions can be seen in Figure 6.3(a) and (b). From these plates can be seen DNS-NH_2, DNS-OH, DNS-e-lysine and DNS-o-tyrosine. From this plate the presence of both lysine and tyrosine in the chromaffin granule DCCD-reactive protein is demonstrated. However no indication of a free N-terminus for the protein can be shown from this work. It is unlikely that proline is the N-terminal amino acid as heavily overloading the plates did not reveal the presence of even a faint proline spot, and although DNS-proline is acid labile it should not be completely destroyed during a 20 hour hydrolysis. The amino terminal could also be tryptophan, as tryptophan is destroyed by acid hydrolysis, or histidine as DNS-histidine comigrated with DNS-ε-lysine in the thin layer chromatography system used. However, as will be seen later, histidine appears to be totally absent from this protein. There is further evidence that the N-terminus is blocked from attempted sequence determination using phenylisothiocyanate, in which no phenylthiohydantoin derivative could be detected (W. Sebald, personal communication). Discussion of the significance of the blocked N-terminus with respect to current theories of organelle biogenesis is deferred to the end of this chapter.

6.3.1. Amino acid analysis

Amino acid analysis of proteins gives a further method of characterisation. The method used for amino acid analysis of the DCCD reactive protein was acid hydrolysis followed by column chromatography, using a three buffer elution system and detection of ninhydrin positive

Amino acid	Hydrolysis time 20 h	70 h	Best integral Value	Published data Margiolash et al. 1961
Asx	7.16	6.99	7	8
Thr	10.98	9.98	10	10
Glx	11.99	11.06	11 or 12	12
Pro	7.15	6.71	7	4
Gly	10.37	9.58	10	12
Ala	6.51	6.06	6	6
Cys	-	-	-	2
Val	3.32	3.16	3	3
Met	2.10	1.91	2	2
Ile	5.25	5.36	5	6
Leu	6.37	5.96	6	7
Tyr	4.05	3.80	4	4
Phe	4.00	4.00	4	4
His	3.63	3.05	3	3
Lys	13.12	12.42	12	18
Arg	2.21	2.11	2	2
Trp	-	-	-	1

Table 6.2. Amino acid analysis of horse heart cytochrome c.

Values are compared to the integral figure of 4 residues for phenylalanine. Best integral figure are obtained by extrapolation of results for threonine to zero time. The 70 h hydrolysis values were used for valine, leucine and isoleucine. As expected serine was totally absent from the hydrolysate. The published data is from the sequence analysis of the protein as determined by Margiolash et al. 1961.

peaks as presented in Chapter 2.

6.3.2. Analysis of cytochrome c

In order to ascertain the reliability of acid hydrolysis and the efficiency of the analyser, a control experiment was performed, in which a protein of known amino acid composition (horse heart cytochrome c) was acid hydrolysed and analysed.

Method. Two 10 nmol samples of cytochrome c (determined spectrophotometrically) were lyophilised in thick walled, acid washed pyrex tubes. 0.3 mls of freshly prepared 6 M HCl was added to each sample and the tubes vacuum-sealed using a vacuum pump and oxygen flame. The tubes were then incubated for 20 h and 70 h at 105^{o}C. After this time the tubes were opened and vacuum desiccated over NaOH flakes, and the hydrolysate resuspended in 100 µl of pH 2.2 buffer for amino acid analysis. Amino acid analysis was carried out and the amino acids determined by use of an automated integrator, or using a manual method of determining the area under individual peaks. The results are presented in Table 6.2. They suggest that the amount of cytochrome c actually used in the hydrolysis was closer to 18 nmoles. Clearly 150 nmoles of each individual amino acid is the maximum that could be determined accurately by the analyser. More than 150 nmoles of an individual amino acid gives quite large underestimates of the amount in the sample, as is the case for lysine in this analysis. An underestimate of threonine and glycine, which are major constituents of this protein, was also found. Apart from proline, the peaks of the lesser amino acid constituents were well resolved and determination of the relative proportion of these give accurate results when compared to the published data (Margiolash et al., 1961). Other points of note from the trace were that glycine-alanine, and leucine-isoleucine peaks were not well resolved.

Amino acid	Automated (nmol)		Manual (nmol)		mols/2 mol Lys		mols/2 mol Lys		Best
	20 h	70 h	20 h	70 h	20 h(A)	70 h(A)	20 h(M)	70 h(M)	Integer
Asx	29.82	26.65	26.92	26.44	3.25	2.84	2.99	2.90	3
Thr	16.92	17.29	19.21	20.47	1.84	1.84	2.13	2.25	2
Ser	104.25	97.05	107.82	97.18	11.36	10.36	11.96	10.68	12
Glu	33.28	29.98	32.37	36.54	3.63	3.20	3.59	4.01	4
Pro	24.19	16.69	17.38	16.68	2.63	1.78	1.93	1.83	2
Gly	81.00	85.31	77.11	75.99	8.82	9.11	8.56	8.35	8
Ala	93.03	103.40	79.00	85.58	10.13	11.04	8.77	9.40	9
Cys	0.00	0.00	1.63	1.83	0	0	0.18	0.20	0
Val	28.39	42.02	28.55	44.09	3.09	4.49	3.17	4.84	5
Met	19.70	5.21	25.17	8.06	2.14	0.56	2.79	0.89	3
Ile	21.25	39.14	26.66	35.71	2.31	4.18	2.96	3.92	4
Leu	50.70	63.30	46.64	60.89	5.50	6.76	5.17	6.70	7
Tyr	11.08	11.59	9.65	10.60	1.21	1.23	1.07	1.16	1
Phe	19.89	26.37	19.20	25.57	2.16	2.81	2.13	2.81	3
His	0.24	2.57	2.86	3.32	0.03	0.27	0.23	0.36	0
Lys	18.36	18.72	18.01	18.19	2.00	2.00	2.00	2.00	2
Arg	16.93	19.01	16.17	18.06	1.84	2.03	1.79	1.92	2

Table 6.3. Results of amino acid analysis of HCl hydrolysed DCCD reactive protein, hydrolysed for 20 and 70 h.

The table shows results obtained using an automatic and manual integration method. (A) = automatic integration (M) = manual integration. The results are also presented as mols per 2 mols lysine, and best integral value, taking 70 h hydrolyses for Val, Leu and Ile, extrapolation to zero time for Thr and Ser. The methionine was taken as the highest result.

6.3.3. Amino acid analysis of the chromaffin granule DCCD-reactive protein

A further characterisation of the DCCD-reactive protein could be achieved by simple amino acid analysis of the purified polypeptide. Comparisons between analyses of different preparations were made, in order to give some indication of the purity of the protein and the reproducibility of the purification procedure.

Method. Chromaffin granule membrane DCCD-reactive protein purified as described in Chapter 5, was stored at $0^{\circ}C$ in chloroform: methanol. Approximately 20 nmoles of protein, as judged from the intensity of coomassie blue staining on SDS polyacrylamide gels, were dried down using a nitrogen stream in each of two acid washed pyrex tubes. 0.3 mls of 6 M HCl was added to each and the tubes evacuated before sealing. The two sealed tubes were incubated for different times (20 hours and 70 hours at $105^{\circ}C$) before breaking the seal and drying the contents by vacuum desiccation over NaOH pellets. The contents were redissolved in pH 2.2 buffer for amino acid analysis. The results are presented in Table 6.3.

A comparison of the results obtained by automatic and manual integration is of interest. The automatic integrator works on the assumption that the amino acid elutes within $\pm$ 1 minute of the standard amino acid run. Colour constants have also been derived from the standards and the amount of each individual amino acid can be worked out from estimates of the peak area. The main criticism of the automatic integrator is that it does not cope well with peaks that elute close to each other so that one peak interferes with another, or with amino acids that for any reason eluted anomalously early or late during the run. The manual integration method involves calculating the areas under the peak and assuming the peaks to be triangular (although the

scale is logarithmic). Manual work also needs calibration by standard amino acid runs, as the different amino acids have different colour constants, which makes the calculations very time consuming. The manual integration makes no allowance for the tails at each peak. However as the area is calculated using the top half of the peak, those peaks which are less well resolved will be determined more accurately. From Table 6.3, there is close agreement between the manual and automated integration for the content of arginine, lysine, phenylalanine, proline, serine and aspartate. The automatic integrator could detect no peak for cysteine. However there was a very small peak eluting in this position. During the run the methionine peak was retarded and the automatic integrator did not recognise it. Values that were only slightly divergent were those for glutamate/glutamine, methionine and leucine. The large discrepancy between the 20 h and 70 h hydrolysis times for leucine, isoleucine, valine and phenylalanine indicates the presence of several dipeptide bonds involving those amino acids.

In addition to these peaks, there were observable on the trace a peak of ammonia, and three other peaks near the end of the trace, corresponding to basic compounds that did not comigrate with any other peaks. However, since the analyser used borate as the third buffer, rather than citrate, it was impossible to compare the results with two useful compilations of analyses which include a large number of different ninhydrin-positive compounds (Hamilton 1963, Adriaens et al. 1977). These workers used citrate as the third buffer rather than borate, which caused the basic amino acids to elute in a different order.

6.3.4. Second amino acid analysis of the DCCD-reactive protein

A second analysis was done on a different preparation of DCCD-reactive protein from chromaffin granule membranes, obtained by a

Amino acid	40 h (nmol) hydrolysis	90 h (nmol) hydrolysis	mols/2 mol lysine 40 h	mols/2 mol lysine 90 h	Best Integer
Asx	88.51	61.13	4.30	3.72	4
Thr	47.30	36.04	2.29	2.20	2
Ser	184.13	156.69	8.95	9.56	9
Glx	74.73	61.51	3.60	3.75	4
Pro	69.16	58.44	3.36	3.56	3
Gly	159.15	139.50	7.74	8.50	8
Ala	162.56	149.96	7.9	9.14	9
Cys	-	-	-	-	-
Val	88.31	101.67	4.29	6.20	6
Met	20.46	26.15	0.99	1.59	1
Ile	66.60	82.45	3.24	5.03	5
Leu	116.58	117.68	5.66	7.17	7
Tyr	29.74	33.64	1.45	2.05	2
Phe	53.59	51.68	2.59	3.15	3
His	8.33	1.07	0.40	0.06	0
Lys	41.14	32.79	2.00	2.00	2
Arg	46.17	35.66	2.24	2.17	2

Table 6.4. Results of amino acid nalysis of DCCD reactive protein hydrolysed for 40 h and 90 h at 105°C in vacuo.

The table contains mols individual amino acids, mols per 2 mols lysine and best integral values for each amino acid. Best values take 90 h hydrolysis for phenylalanine, valine, leucine and isoleucine, and extrapolation to zero time for threonine and serine.

purification procedure which included water extraction of the LH20 column eluate. The protein was hydrolysed as described in 6.3.3. except that the hydrolysis times were 40 hours and 90 hours instead of 20 hours and 70 hours and a new estimate of the relative amounts of isoleucine, leucine and phenylalanine would be obtained. The results are presented in Table 6.4.

The values obtained agree fairly well with the initial analysis except that the relative methionine values are decreased. This may well be due to improper evacuation of the pyrex tubes before sealing, leading to destruction of methionine. The relative amounts obtained for valine, leucine, isoleucine and phenylalanine appear to be much greater with the 90 hour hydrolysis reaching a nearly integral value when compared to lysine. The reduced amount of serine present could be either due to inaccuracies in extrapolation to zero time or possibly to removal of some of the serine (as phosphatidyl serine) during washing of the protein with water.

These results suggest that the preparation procedure for the protein is repeatable, and the negligible amounts of histidine suggest little contamination by other proteins.

6.3.5. Determination of cysteine and methionine

Cysteine is almost entirely destroyed by acid hydrolysis of proteins and methionine is highly susceptible to oxidation if there is incomplete evacuation of tubes before sealing. Conversion of these amino acids to stable products before analysis is one of the recognised methods of overcoming this problem. The method used here is to treat the protein with performic acid prior to acid hydrolysis of the protein (Spackman *et al.* 1958). This converts all of the cysteine to cysteic acid and the methionine to methionine sulphone. These derivatives can be detected on the amino acid analyser.

Amino acid	(nmol) amino acid	mols/2 mol lysine	Best Integer
Asx	44.55	3.25	3
Thr	30.43	2.22	2
Ser	131.39	9.59	10
Glx	59.30	4.33	4
Pro	48.78	3.56	3
Gly	107.49	7.85	8
Ala	111.36	8.13	8
Cys	1.64	0.12	0
Val	58.86	4.29	4
Met	38.88	2.84	3
Ile	47.24	3.45	3
Leu	79.60	5.81	6
Tyr	21.25	1.55	2
Phe	37.08	2.72	3
His	1.22	0.09	0
Lys	27.38	2.00	2
Arg	29.26	2.13	2

Table 6.5. Amino acid analysis of performic acid oxidised protein.

14 nmols of DCCD-reactive protein was oxidised, and acid hydrolysed as described in the text, using automatic, and manually integrated figures. Methionine and cysteine were determined as the sum of methionine, and methionine sulphone, cysteine and cysteic acid.

Method. 10 nmoles of a chloroform:methanol solution of DCCD-reactive protein were dried in a stream of nitrogen. The purification procedure was the same as described in section 6.3.4. To this protein was added 0.1 mls of performic acid, made by mixing one volume 30% H_2O_2 with nine volumes of formic acid. The protein was left in performic acid ($0^{o}C$) for one hour, the tube and contents lyophilised and the lyophilised material resuspended in 0.3 mls of 6M HCl. The protein was hydrolysed in sealed evacuated tubes for 40 hours. The seal was then broken, the contents dried by vacuum desiccation over NaOH flakes, and redissolved in analyser buffer (pH 2.2). Amino acid analysis was carried out as described in Chapter 2. The colour constant for cysteic acid was taken as 1.01 times that of aspartate, and for methionine sulphone, as identical to aspartate (Spackman et al. 1958). The results of this analysis are presented in Table 6.5. As can be seen from this table, cysteine appears to be totally absent from this protein whilst methionine values of three per two lysines have been confirmed. The reasons for choosing two moles of lysine per mole of protein are not apparent from evidence presented so far. First, lysine is one of the most stable of the amino acids during acid hydrolysis of protein. Second, the lysine peak is well resolved. Using 2 moles lysine/mole protein gives values close to integers for the other amino acids eluted in the analysis. If values of nine moles/mole of alanine or eight moles glycine/mole protein were chosen values for the individual amino acids were no closer to integral values than use of 2 lysines/mole of protein. Finally the value of 2 moles lysine/mole protein gives a molecular weight slightly lower than that of the mitochondrial protein, in agreement with the observed electrophoretic mobility on SDS polyacrylamide gels, which is slightly higher than that of the mitochondrial protein. This is the best estimate of molecular weight that can be obtained for

Amino acid	nmoles in analysis (nmol)	moles/2 moles Lys	Best Integer
Asx	84.84	3.42	3
Thr	73.99	2.98	3
Ser	155.64	6.28	6
Glx	82.49	3.33	3
Pro	74.45	3.00	3
Gly	182.14	7.35	7
Ala	211.15	8.52	9
Cys	4.15	0.09	0
Val	111.96	4.52	5
Met	73.30	2.95	3
Ile	129.55	5.23	5
Leu	160.32	6.46	6
Phe	75.31	2.62	3
His	1.03	0.02	0
Trp I	14.56	0.58	)
Trp II	10.43	0.42	) 1)
Tyr	65.04	2.62	3
Lys	49.54	2.00	2
Arg	53.46	2.15	2

Table 6.6. Amino acid analysis of the DCCD-reactive protein from chromaffin granules using MESA

As the analyser was deliberately overloaded, to give significant values for the content of tryptophan, accurate results can only be obtained for the less common amino acids in this analysis.

the protein at the present time.

6.3.6. Hydrolysis of the protein with mercaptoethane sulphonic acid (MESA)

In the experiments described so far, it was impossible to measure the tryptophan content of the DCCD-reactive protein; nor was this possible by absorbance measurements using the protein as prepared in chloroform:methanol. The spectrum of the chloroform:methanol extract shows a high absorbance at 260 nm which completely obscures any peak at 280 nm, as shown in Figure 5.6(b). Use of methane sulphonic acid to preserve tryptophan upon hydrolysis in carefully controlled conditions (Moore, 1972) has been successful, giving good yields of tryptophan. More recently MESA has been used to estimate the tryptophan content of proteins (Penke et al., 1974) with greater efficiency in preserving tryptophan than methane sulphonic acid (Allen, 1980).

Method. 25 nmoles of DCCD-reactive protein, purified from chromaffin granule membranes, and stored in chloroform:methanol was extracted three times with water and precipitated three times with acetone. The protein was gently dried in a nitrogen stream, suspended in 100 µl MESA (3 M) and vacuum sealed in acid washed pyrex tubes. The sealed tubes were incubated for 120 hours (105^{o}C) in order to fully hydrolyse the protein. The tube was opened and the contents neutralised with freshly prepared NaOH prior to analysis. The colour constant for tryptophan, and its major breakdown product was taken as 78% of that for lysine (Liu and Chang, 1971). The results of the amino acid analysis are presented in Table 6.6. It can be seen from this table that the more common amino acids have been overloaded with values of greater than 150 nmoles being obtained: consequently the contents of these are underestimated. The reason for heavily overloading the trace

was that it gives a more accurate assessment of tryptophan, which eluted in a double peak, one part of which has been shown to be due to an acid breakdown product (Glazer et al., 1975). A similar result was obtained when glycyl-tryptophan was analysed by the same procedure (Chapter 2). The value obtained gives one tryptophan per two lysine. The other feature of MESA analysis is that the methionine is undegraded, so that the analysis gives a further check on the methionine content of the protein. It is likely to be a more accurate assessment than the performic acid oxidation as methionine itself is eluted well away from other major amino acid peaks, whereas methionine sulphone is eluted very close to serine and aspartate on this analyser.

6.4.1. Discussion of N-terminal analysis

Lack of a recognisable N-terminal amino acid in the chromaffin granule DCCD reactive protein, as judged both by dansyl-chloride treatment in SDS and by lack of a phenylthiohydantoin product upon attempted Edman degradation, shows that the N-terminus is blocked. This result is rather unexpected, as if the protein is synthesised on cytoplasmic ribosomes and inserted directly into the membrane of the endoplasmic reticulum, there is no obvious reason for the modification of the N-terminus. Of the DCCD-reactive proteins isolated from various other organelles, it has been shown that the only proteins with a blocked N-terminus are those of chloroplasts and *S. cerevisiae* mitochondria, both of which are synthesised on intraorganellar ribosomes. In these cases the N-terminus is always N-formyl-methionine, the initiator of translation in mitochondria and chloroplasts (Feldman and Mahler, 1974). This, coupled with the demonstration of no deformylase within the mitochondrion is the reason why intra-organellar synthesis of proteins always results in N-formyl-methionine as the N-terminal amino acid. The

proteins isolated from the bacterial plasma membrane H^+ translocating ATPases also have an N-terminus of N-formyl-methionine as it is the initiator of bacterial protein synthesis, and the similarities between protein synthesis in prokaryotes, chloroplasts and mitochondria tend to support the speculation that these organelles originated during the symbiosis of bacteria with eukaryotic cells. This endosymbiosis must have occurred with very primitive bacteria as the genetic code in mitochondria is very similar but not identical to the universal code (Barrell et al., 1979). It is unlikely that the chromaffin granule DCCD-reactive protein has formyl-methionine as its N-terminus, as it is presumably the product of protein synthesis by ribosomes bound to the endoplasmic reticulum. This raises the question of what group could be blocking the N-terminus. As discussed it is unlikely that the blocking group is a formyl residue; also unlikely is that the amino terminus is blocked by glycosylation of the protein, as the protein shows no sign of being stained with various carbohydrate stains following SDS polyacrylamide gel electrophoresis (Abbs, Ph.D. thesis, 1980, Abbs and Phillips 1980): there is certainly no precedent for glycosylation of the N-terminus (Allen, 1980). The DCCD-reactive protein (band 61 of Abbs and Phillips, 1980) stains faintly with the lipid stain Sudan Red IV and it is possible that the N-terminus is modified by the attachment of lipid. One protein already isolated with N-terminal fatty acyl blocking group is a murein lipoprotein from bacterial cell wall (Hantke and Braun, 1973). Phosphatidyl serine has been shown to be the N-terminus of a penicillinase (Yamomoto and Lampen, 1975) but this would be detected by the dansylation procedure used.

More likely is that the amino terminus is acetylated as has been shown to occur in many eukaryotic proteins including cytochrome b_5 (Ozols, et al., 1976) cytochrome b_5 reductase (Mihara et al., 1978) cytochrome c

Amino acid	No. of residues per protein
Asx	3
Thr	2
Ser	9
Glx	4
Pro	3
Gly	8
Ala	9
Cys	0
Val	5
Met	3
Ile	5
Leu	7
Phe	3
His	0
Trp	1
Tyr	2
Lys	2
Arg	2
TOTAL	68
HYDROPHOBICITY	31.9%

Table 6.7. Amino acid content of the Chromaffin granule DCCD-reactive protein.

The table presented a compilation of data presented in Tables 6.3-6.6 and the best result for each individual amino acid is taken. Hydrobicity (polarity) is calculated by the method of Capaldi and Vanderkooj (1972) as the % polar amino acid residues.

(Margiolash et al., 1961) and rabbit sarcoplasmic reticulum Ca^{2+} ATPase (Tong, 1977); both the reductase and the Ca^{2+} ATPase are membrane bound. Another common blocking group is the pyroglutamyl residue shown to block the N-terminus of hog amylase (Kluh, 1979) as well as many other proteins. These modifications are difficult to detect; methods used are hydrazinolysis to detect acetyl groups, which requires anhydrous hydrazine, or proteolytic digestion of the protein, followed by a search for a peptide with a blocked N-terminus.

6.4.2. The amino acid analyses

A compilation of the amino acid analyses presented in Tables 6.3-6 is presented in Table 6.7. From this table the number of residues is 68 per protein and the minimum molecular weight 7008. When comparing Table 6.7 with Table 6.1 several interesting points emerge. The DCCD-reactive protein of chromaffin granules lacks histidine and cysteine: histidine is also absent from all other DCCD-reactive proteins so far studied, and cysteine is present in only a few. One of the most interesting features to emerge is the presence of a single tryptophan residue in the protein. In this the chromaffin granule protein differs from all the DCCD-reactive proteins so far studied. It is of interest to note that the codons for tryptophan are UGA and UGG in the mitochondrial genome (Barrell et al., 1979): the former is read as a stop codon in other systems. Transfer of a given gene from the mitochondrial genome into the nuclear genome would be hindered by this and other differences in the genetic code. However such a transfer appears to have occurred in some cases, and the absence of tryptophan may be a prerequisite for this; the DCCD-reactive protein (subunit 9 of the H^+ translocating ATPase from mitochondria) is a mitochondrial gene product in yeast, but is made in the cytoplasm of N. crassa and of animal cells (Sebald et al., 1978, Aroskar and Avadhani, 1980). Of course these constraints do not apply

to the chromaffin granule protein, and the presence of tryptophan in the granule protein does not contradict the arguments just presented. However, it does argue against the absence of tryptophan having something to do with the proteins' function in proton conduction. It could well be that the absence of histidine is due to a functional property of the DCCD-reactive proteins, as histidine has been shown to be present in other organic solvent soluble proteins isolated from other ion translocating ATPases.

The overall amino acid composition is very similar to that of other DCCD-reactive proteins in the high proportion of hydrophobic amino acids present and although hydrophobicity calculations give a higher hydrophobicity for other DCCD-reactive proteins; that is 31.9% for the granule protein and 16.5-24.7% for other proteins (Sebald et al., 1979(b)), this is affected by the content of serine which could be due to contamination of the protein with phospholipid. However reducing the serine content to six per protein only reduces the hydrophobicity to 29.1% using the system of Capaldi and Vanderkooj (1972) to calculate hydrophobicity. It is also noteworthy that this protein has fewer amino acid residues than any so far isolated.

The single undefined peak in amino acid analyses eluting between tryptophan and lysine may indicate the presence of a modified amino acid or to a contaminating ninhydrin-reactive compound. Experiments with phosphatidyl-ethanolamine shows that it runs as a shoulder on the trailing edge of the ammonia peak. As the undefined peak is still present after 96 hours of hydrolysis it is unlikely to be due to dipeptides. Other contaminants could be catecholamines, or some contaminant of the organic solvents used during the purification procedure.

6.5. Summary

1. N-terminal analysis of the chromaffin granule DCCD-reactive protein suggests that this residue is modified.

2. The amino acid composition is similar to that isolated from other DCCD-reactive proteins, in its lack of cysteine and histidine; however, in contrast, the presence of a single tryptophan residue was demonstrated.

3. These results are discussed in terms of current knowledge of protein synthesis in cellular organelles.

Chapter 7

Isoelectric focussing of chromaffin granule membranes

7.1 Introduction

At present very little work has been published on two dimensional electrophoretic characterisation of chromaffin granule proteins. The method used here is based upon that of O'Farrell (1975), which utilises separation by isoelectric focussing (IEF) in tubular gels in the first dimension, followed by discontinuous SDS polyacrylamide gel electrophoresis in the second.

Early work using IEF to separate the proteins of the chromaffin granule matrix (Baumgartner, 1974) indicated that these soluble proteins were clustered between isoelectric points (pI) of 4.8-5.6 with a minor band at pI = 7.1. However Koenig (1974) reported conflicting results, in that the pIs of the soluble proteins were between 3.9 and 4.5. Apps et al. (1980c) using two dimensional gel electrophoresis of the soluble components obtained results that concurred with those of Baumgartner.

The latter authors appear to be the only ones to have attempted two dimensional gel electrophoresis of any membrane proteins from chromaffin granules. Using Nonidet P40 to solubilise the membranes, followed by IEF and discontinuous slab gel electrophoresis, Apps et al. identified the positions of chromogramin A, cytochrome b_{561} and dopamine β-hydroxylase. pIs for these proteins were shown to be 4.5, 6.2 and 5.9 respectively.

In this chapter the initial aim was to obtain pI values for the DCCD-reactive proteins of beef adrenal mitochondria and chromaffin granules. The only previously reported work on IEF of any DCCD-reactive proteins is that of Aroskar and Avadhani (1979). They showed a rather low pI for the mouse liver proteolipid of 5.9. However these authors gave no indication of whether the amido black staining component in their gel system bound DCCD or migrated in a second dimension with an

electrophoretic mobility consistent with the expected molecular weight of 7800.

7.2. Two dimensional electrophoresis of chromaffin granule membranes and adrenal mitochondrial membranes.

This technique, based upon that of O'Farrell (1975), provides high resolution of membrane proteins. The method used here differs in many details from that described by O'Farrell and warrants a full description here.

7.2.1. Pouring and electrophoresis of electrofocussing gels.

Glass tubes (internal dimensions 0.3 x 14 cm) were washed by immersion in chromic acid. After thorough rinsing, the tubes were dried by heating and sealed at one end with parafilm. The best seal was formed if the bottom of the tube was sealed whilst the tube was still warm and a second layer of parafilm added when the tube had cooled. Gel mixture (enough for five tubes) contained 2.38 g urea (BDH aristar grade), 0.41 mls of acrylamide:NN'-methylene bisacrylamide (45%:0.6% w/v), 0.8 mls of 10% w/v nonionic detergent, 1.16 mls of twice distilled water, and 0.2 mls of the ampholine mixture to be used. The solution was then swirled until the urea had dissolved (when $C_{12}E_8$ was used the solution required warming in order to fully dissolve the constituents). The solution was then degassed, care being taken to avoid frothing.

20 µl ammonium persulphate (10% w/v) was added, the solution taken up into a syringe and poured into each tube using a long needle placed just above the parafilm. The final length of the gel was 11.5 cm. After pouring the top was flattened by the addition of water saturated butanol. These steps had to be performed with haste as the solution polymerised five to ten minutes after the addition of ammonium

persulphate. As soon as polymerisation was complete the butanol was removed and replaced with sample buffer.

7.2.2. Preparation of samples for IEF.

Best results were achieved using freshly-prepared membranes. Precipitation of the proteins with TCA or acetone:ethanol (1:1) to remove lipid resulted in a greatly decreased proportion of the proteins entering the tube gel and so was not used. As mentioned by Apps et al. (1980c), a considerable proportion of the 'soluble' proteins remain attached to the membrane. Since these proteins were readily solubilised by Nonidet P40 treatment of the membranes, whereas intrinsic membrane proteins were not, they tended to dominate the two dimensional "maps" of the chromaffin granule membranes. Osmotic shock, by use of Hepes and sucrose / Hepes buffers could release some of these proteins. Use of Tween 20 to remove extrinsic proteins resulted in streaking of the proteins as seen in subsequent staining patterns.

The best two dimensional "maps" of the chromaffin granule membranes were produced when 150 µg protein were applied whilst for mitochondria up to 200 µg protein could be used. Since 100 µl was the maximum volume which could be loaded into the tubes membrane protein was suspended at 6 mg/ml (chromaffin granules) and 8 mg/ml (mitochondria) and diluted to the required concentration by the addition of sample buffer. Sample buffer was prepared from 0.57 g urea, 0.2 mls of 10% w/v nonionic detergent, 50 µl ampholine mixture, 50 µl 2-mercaptoethanol, 0.3 mls water and approximately 5 µg/ml bromophenol blue. Addition of small amounts of SDS (0.1% w/v final concentration) to the sample buffer, which has been reported to increase the solubilisation of membrane proteins from other sources resulted in streaking of the proteins during focussing, and was therefore not used for chromaffin granule membranes.

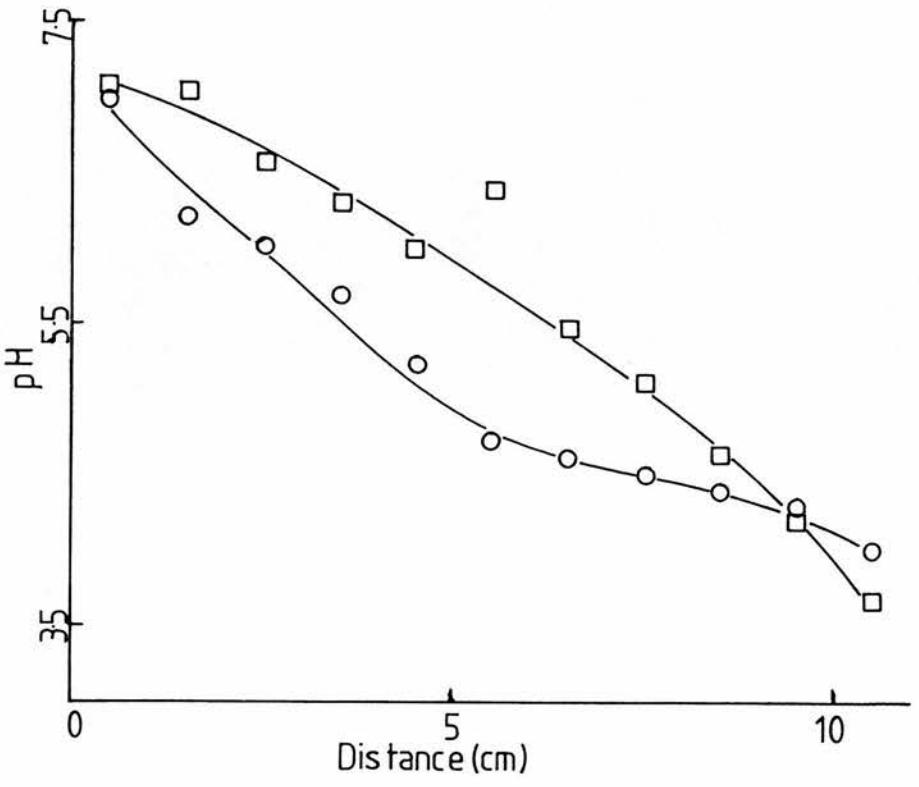


Figure 7.1. pH gradient formed after isoelectric focussing (1).

The diagram shows the pH gradient formed using LKB wide range 3.5-10 (□) or Biolyte pH 4-9 (o) ampholine focussed, as described in Chapter 7.2, with acid solution in the bottom tank.

7.2.3. Choice of detergent for electrofocussing.

The original technique (O'Farrell, 1975) used Nonidet P40 as the nonionic detergent, and in the present work it gave adequate "maps" of both mitochondria and chromaffin granules. However (see chapter 4) Nonidet is by no means the best detergent at solubilising the chromaffin granule membrane and so IEF was attempted with the following detergents: Brij 36T, Triton X-100, β-octylglucoside, Nonidet P40 and $C_{12}E_8$. For both membranes the use of Triton X-100 resulted in streaking of the proteins in the first dimension, and Brij 36T gave a very unusual focussing pattern with all the proteins clustering at around pH 6-6.5. β-octylglucoside was unsatisfactory for chromaffin granules, only a small fraction of proteins entering the IEF tubes, whilst for adrenal mitochondria excellent results, both in sharpness of focussing and entry of proteins, were obtained. $C_{12}E_8$ on the other hand yielded poor results for the mitochondrial membranes and the best obtainable results for chromaffin granule membranes. It should be noted that no detergent tried produced better solubilisation than $C_{12}E_8$ with respect to the subsequent IEF "maps".

7.2.4. Type of ampholines

Three types of ampholine were used: LKB wide range 3.5-10 and narrow range 9-11, and Biolyte pH range 4-9 (Biorad). Figures 7.1 and 7.2 show the pH gradients formed by the Biolyte and LKB wide range ampholines. These were measured by slicing the tube after focussing was complete into 1 cm lengths, eluting the ampholines into 1 ml water and measuring the pH. Since the proteins were loaded in a solution containing ampholines, the pH gradient was formed throughout the gel and the overlaying protein solution, and the top end of the gradient was lost after electrophoresis. Also since focussing could be performed with acid in the top or bottom tank (see later) the pH gradient formed

depended on the direction of the applied voltage. The gradients shown in Figure 7.1 are for IEF tubes electrophoresed with the acid solution in the bottom (anodal) tank whilst Fig. 7.2 shows gradient formed with acid in the top tank. It can be seen from both of these figures that the LKB wide range ampholine gave a smoother gradient than "Biolyte 4-9" and so the former was chosen for subsequent experiments.

In order to try and improve the gradient the effect of adding some LKB pH range 9-11 ampholine was tried. Two different ratios of pH 9-11:3.5-10 ampholines were used; these were 1:9 and 3:4 and the pH gradients formed with these mixtures run with the anode at the bottom are shown in Fig. 7.3. Changing the ratio from 1:9 to 3:4 appeared to change the shape of the gradient, making the pH transition above pH 5.2 smoother, but did not markedly alter the highest pH formed in the tube. With anode at the top the effect of adding 9-11 to the mixture was to increase the maximum pH in the tube from 8.4 to 9.0. In all experiments discussed below a 1:9 ratio was used.

7.2.5. Size and direction of applied potential.

Since the method used here was equilibrium isoelectric focussing the tube could be focussed with either the acid solution (0.5% ortho-phosphoric acid) in the top or bottom tank, whilst the cathode (basic) solution (1% ethylenediamine) was in the other tank. This was found to be quite important and for chromaffin granule membranes the best results were achieved with the anode at the bottom end, whilst for mitochondrial membranes good focussing "maps" were obtained with the anode in the top tank.

The potential was applied in a series of stages: one hour at 100v, thirty minutes at 200v, twelve hours at 300v and one hour at 500v.

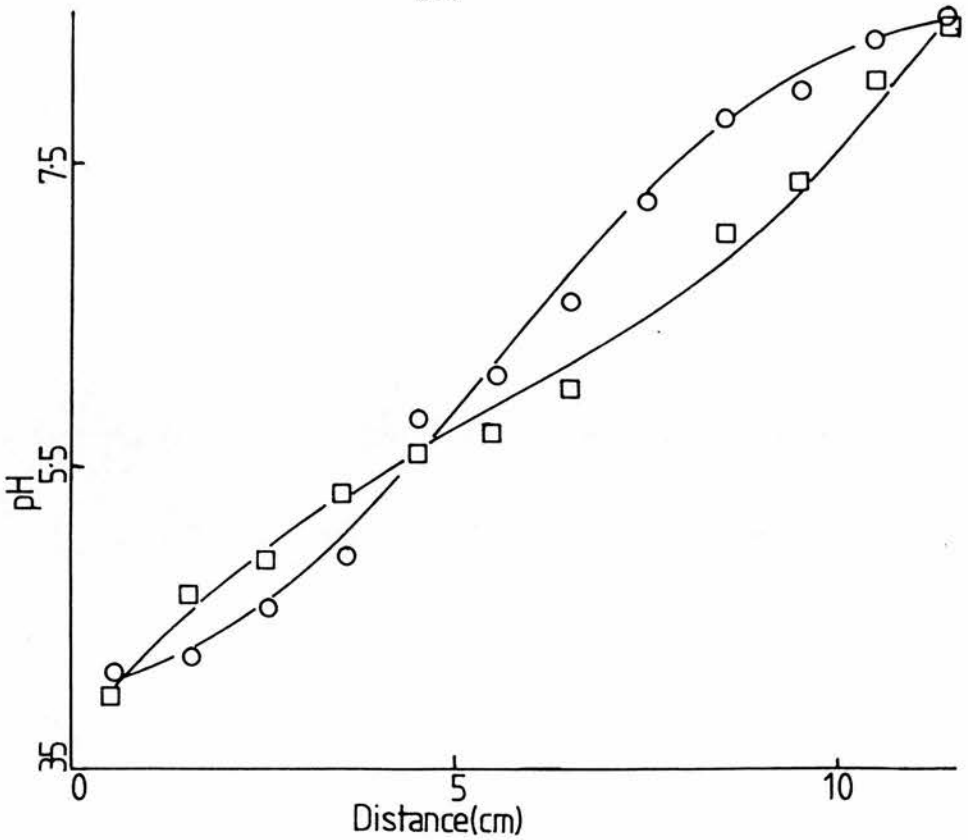


Figure 7.2. pH gradient formed after isoelectric focussing (2).

The diagram shows the pH gradient formed using LKB wide range 3.5-10 (□) or Biolyte pH 4-9 (o) ampholines focussed as described in Chapter 7.2 with acid solution in the top tank.

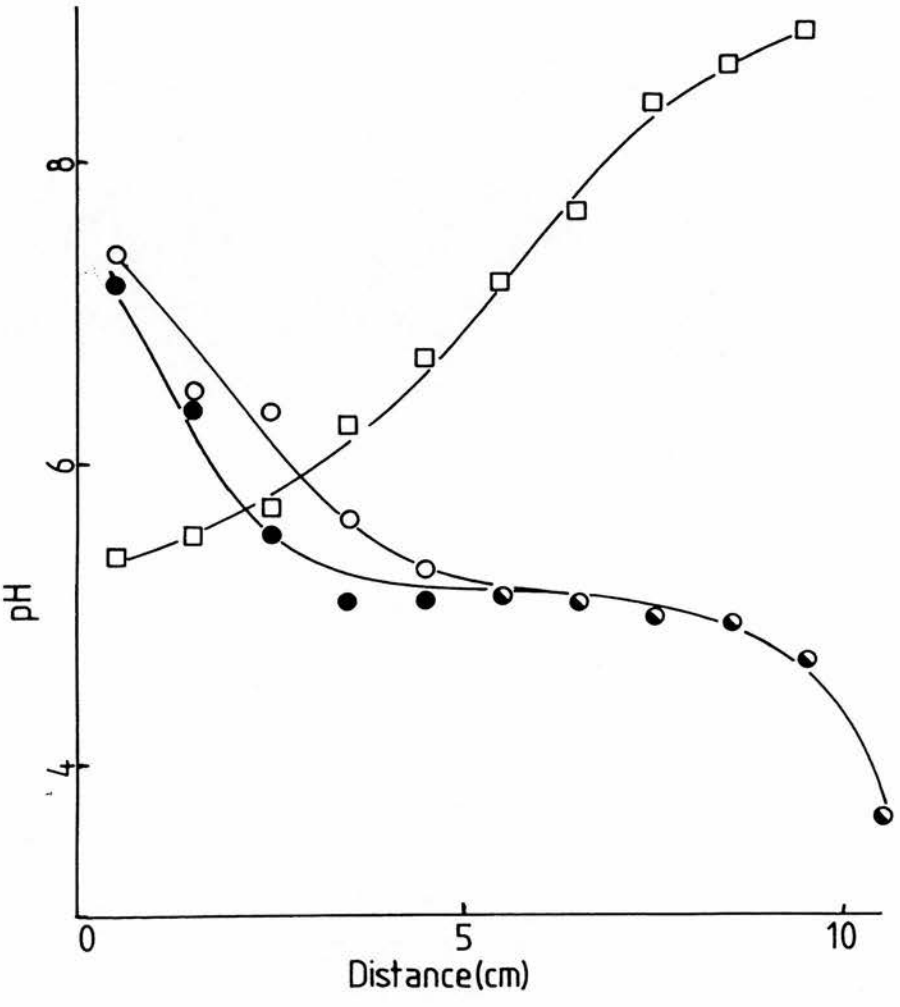


Figure 7.3. pH gradient formed after isoelectric focussing using mixtures of ampholines.

The diagram shows the pH gradient formed using a mixture of LKB wide range 3.5-10 and LKB narrow range pH 9-11, ampholines used in the following ratios 9:1 (●,□) and 3:4, focussed with acid solution in the top (□) or bottom (○,●) tank.

7.2.6. Removal of gel and second dimension electrophoresis.

After IEF was complete the gel was removed from the tubes using a water filled syringe and long needle, releasing water gently whilst working the syringe needle between the tube and the gel. When the gel was loose it was dropped into a solution containing 3% w/v SDS, 3% 2-mercaptothenaol, 50 mM Tris HCl pH 6.8, 10% v/v glycerol ("equilibration buffer") for 10 minutes. The tube was then fixed to the top of a discontinuous 10-15% exponential gradient SDS polyacrylamide gel, poured as described in chapter 2. The method for sticking the tube gel to the top of the slab gel was to fill the slab gel cassette to the top with a hot 1% solution of agarose in equilibration buffer. The focussing gel was laid across the top of the slab, covered with a layer of agarose, and a second layer of agarose added to affix the tube firmly. Care was taken to avoid bubbles during this procedure.

The gel was then electrophoresed, fixed, stained and destained as described in chapter 2. An alternative fixing procedure was to use 25% v/v isopropanol, 10% v/v acetic acid, 65% v/v water; staining was in 27% isopropanol, 10% v/v acetic acid 0.4% Coomassie brilliant blue R250, 0.5% $CuSO_4$, and 0.1% crocein scarlet for 30 mins; destaining in 7% v/v acetic acid, 12% v/v isopropanol, and 0.5% w/v $CuSO_4$ (Blakesly and Boezi, 1977).

7.3 Results.

Chromaffin granule and adrenal mitochondrial membranes were prepared as described in chapter 2, labelled with 20 μM ^{14}C-DCCD in the presence of ATP as described in 3.2.8, dissolved in $C_{12}E_8$- and β-octylglucoside-containing sample buffer respectively, and subjected to IEF as described earlier. Photographs of the stained gels are presented in Fig. 7.4 and 7.5. Figure 7.6 and 7.7 show autoradiograms

base acid

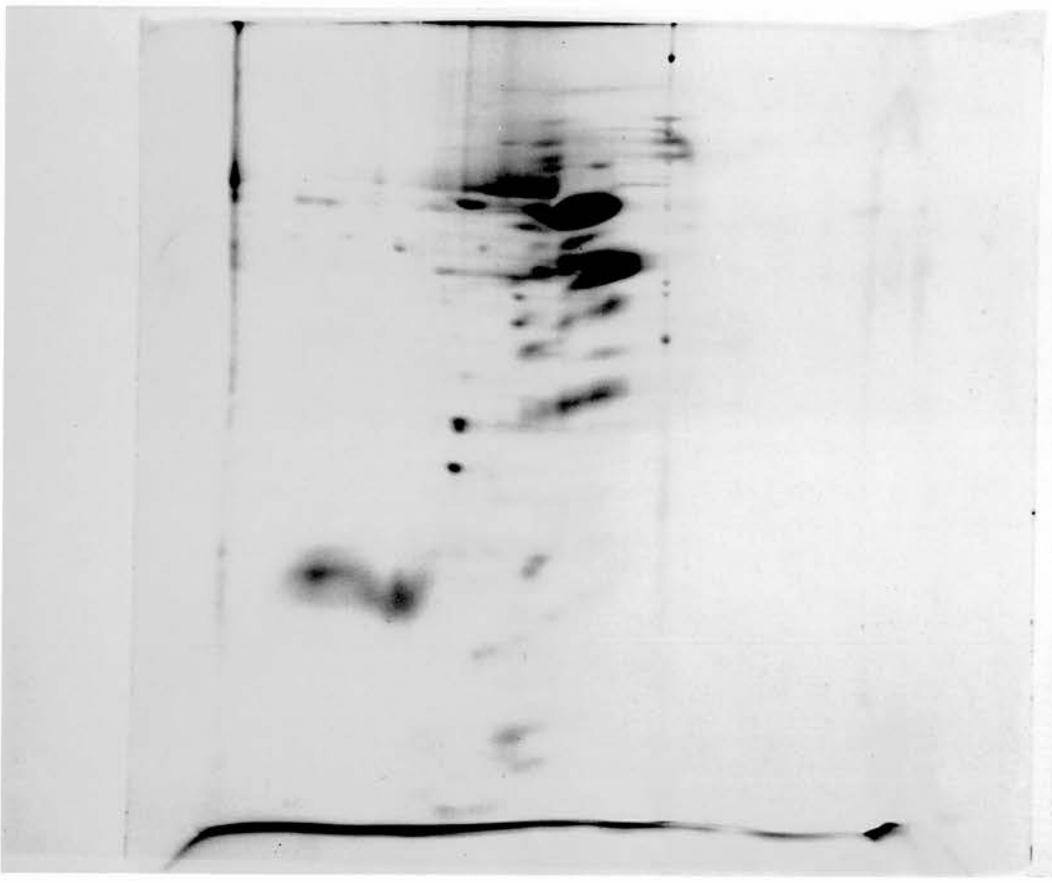


Figure 7.4. Two dimensional electrophoretogram of chromaffin granule membranes.

Conditions are described in the text and the electrophoretogram stained for protein with Coomassie blue. For identification of polypeptides see Apps et al. 1980(c) Figure 4, for comparison.

acid base

Figure 7.5. Two dimensional electrophoretogram of adrenal mitochondrial labelled with ^{14}C-DCCD.

Conditions are described in the text and electrophoretogram stained using the method of Blakesly and Boezi (1977).

acid base

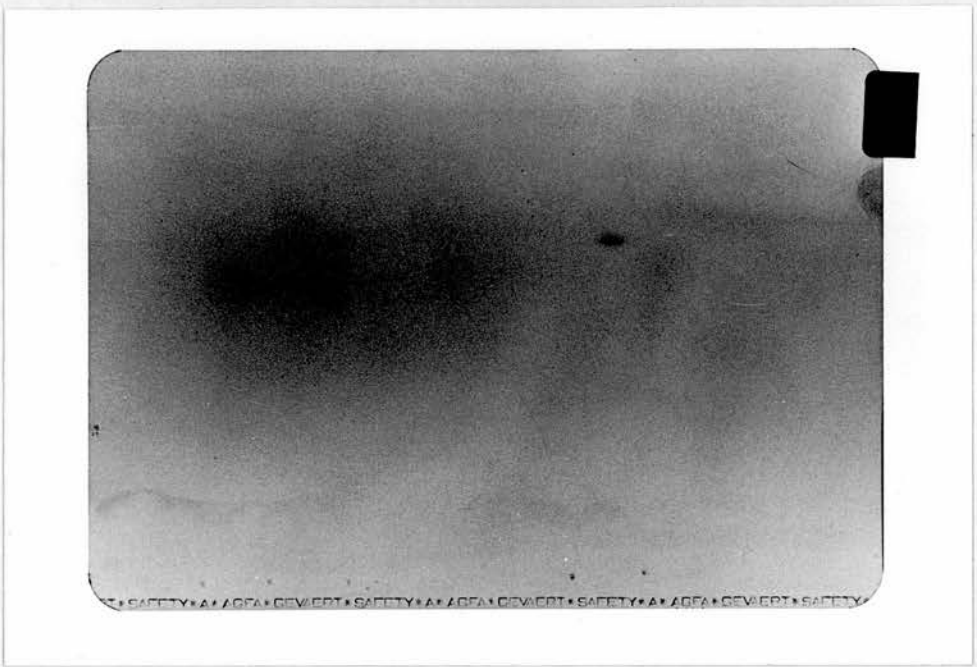


Figure 7.6. Autoradiograph of two dimensional electrophoretogram of chromaffin granule membranes labelled with ^{14}C-DCCD.

Condition for isoelectric focussing are described in the text. The gel was subsequently fixed, impregnated with salicylate and autoradiographed.

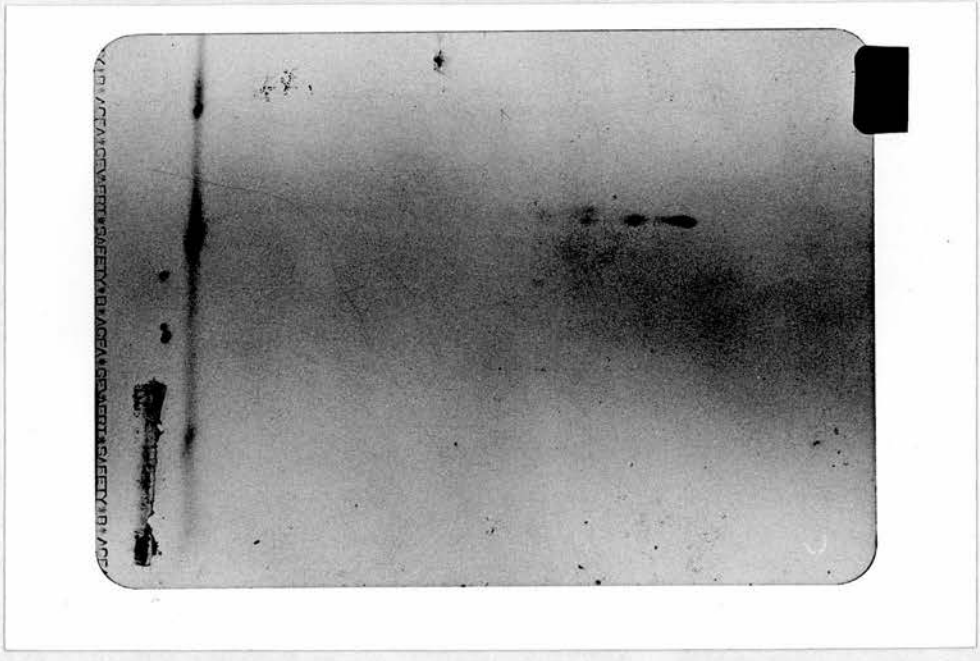


Figure 7.7. Autoradiograph of two dimensional electrophoretogram of adrenal mitochondrial membranes labelled with ^{14}C-DCCD.

The autoradiograph was obtained using the polyacrylamide gel shown in Figure 7.5.

of typical IEF maps of both chromaffin granule and mitochondrial membranes labelled with ^{14}C-DCCD.

From Figures 7.4 and 7.6 several observations can be made. In Fig. 7.6 the low molecular weight DCCD-reactive protein is absent from the entire autoradiogram. The clearly visible spot on the autoradiogram coincided with cytochrome b_{561} on the stained gel (not shown). The DCCD reactive protein, therefore, appears not to enter either the first or second dimension gels. The streak running horizontally across the bottom left hand corner of the autoradiogram comigrated with the dye front on the stained gel.

Attempts to detect the DCCD-reactive protein in two dimensional gels after use of different ampholines, by loading up to 20 µg of purified DCCD reactive protein onto isoelectric focussing gels or focussing in the absence of urea also failed to reveal the presence of any labelled or stained spots. This is probably due to the proteins hydrophobic nature, rendering it insoluble in the urea/nonionic detergent mixture used: and since the DCCD-reactive protein is only barely soluble in SDS it may not electrophorese in the second dimension. Calculations of the pI of the protein, based upon amino acid analysis (chapter 6) and estimates of free carboxyl residues (chapter 5), which give only three carboxyl residues on the protein, gives a value of 8.8 for the DCCD-modified protein (assuming the blocking of a single carboxyl residue). This would not have been within the pH range of even the pH 9-11 IEF gels used in some experiments (not shown), as the high pH end of the pH gradient is consistently lost as the sample was loaded in a solution containing ampholines which are lost after the first dimension.

The staining pattern of chromaffin granule membranes, Fig. 7.4, can be compared with the results of Apps et al. (1980c). The pI values

for the soluble proteins agree well with previous estimates. Cytochrome b_{561} often focussed in the diffuse pattern shown in Fig. 7.4 rather than the spot obtained by Apps et al. (which was however, observed in some experiments such as the autoradiogram). The pI for this protein of 6.1-6.4 also agrees with the previous authors' estimates. The overall impression is that the pattern obtained on two dimensional electrophoresis of chromaffin granule membrane proteins was rather variable, with appearance and disappearance of minor spots.

With mitochondrial membranes (Fig. 7.5 and 7.7) the results were in marked contrast. Although a substantial proportion of the proteins appeared not to enter the first dimension gel (as is suggested by the heavily staining streak of proteins down the left hand side of Figure 7.5), those that did, focussed in sharp spots and charge isomers of some individual polypeptides were apparent. The pattern was highly repeatable, and although only a few spots were clearly identifiable (such as the α and β subunits of the ATPase, the adenine nucleotide translocator and the phosphate transporter) most of the other spots formed a clearly identifiable pattern. Work using antibody techniques and purified complexes might yield a better understanding of this sort of two dimensional map.

The autoradiograph of the gel shown in Fig. 7.5 (Fig. 7.7) was also notable for the absence of any DCCD-reactive protein. The pI of the mitochondrial DCCD-reactive protein, calculated from sequence data, is 8.0, allowing for the modification of a single carboxyl by DCCD. This was certainly within the range for the IEF gels as these gels were focussed with the acid solution in the top tank. Most likely the protein irreversibly aggregates in urea/nonionic detergent fails to enter the first dimension and is not solubilized during electrophoresis in the slab gel. Clearly visible spots in the autoradiogram included the

DCCD-reactive protein (mol. wt. 33 000), recently identified as the phosphate transport protein (Houstek et al., 1981) which has an apparent pI = 5.5 and the β-subunit of the ATPase, known to bind DCCD (Pou geious et al., 1979) whose presence on the autoradiograph is only visible in the streak down the left hand side of the autoradiogram.

7.4.1.Non-denaturing two dimensional gel electrophoresis

All attempts to focuss the DCCD-reactive protein in the presence of chaotropic agents have been unsuccessful, probably due to the hydrophobic nature of this protein. In the next series of experiments, chromaffin granule membranes were solubilized under non-denaturing conditions, by nonionic detergents in the absence of urea. The aim of this experiment was to isolate an active ATPase complex.

7.4.2. Methods: pouring IEF tubes

The gel mixture contained 5.5% w/v acrylamide 0.073% w/v N,N'-methylene bis acrylamide, 2% w/v $C_{12}E_8$, 10% v/v glycerol, 1% w/v ampholines and 0.00025% ammonium persulphate. Tubes were cleaned, sealed and poured as described in 7.2.2, but the samples were prepared differently to the method described in 7.2.5. Freshly prepared chromaffin granule membranes were incubated with 20 μM ^{14}C-DCCD 10 mM ATP, 0^{o}C, for six hours. A final concentration of 1 mg protein.ml^{-1} in the sample buffer was used, the membranes being dissolved in 2% w/v $C_{12}E_8$, 10% v/v glycerol, 1% w/v ampholines either in the presence or absence of 3% 2-mercaptoethanol. The solution was then centrifuged at 25^{o}C (Beckman Ti 50 rotor, 50 000 r.p.m., 30 mins) in order to pellet unsolubilised membranes. After centrifugation a small pellet could be seen, whilst the solution separated into a cloudy infranatant, under which was a clear solution. Both the infranatant and solution were used in IEF, 100 μl being loaded into each tube. Gels were electro-

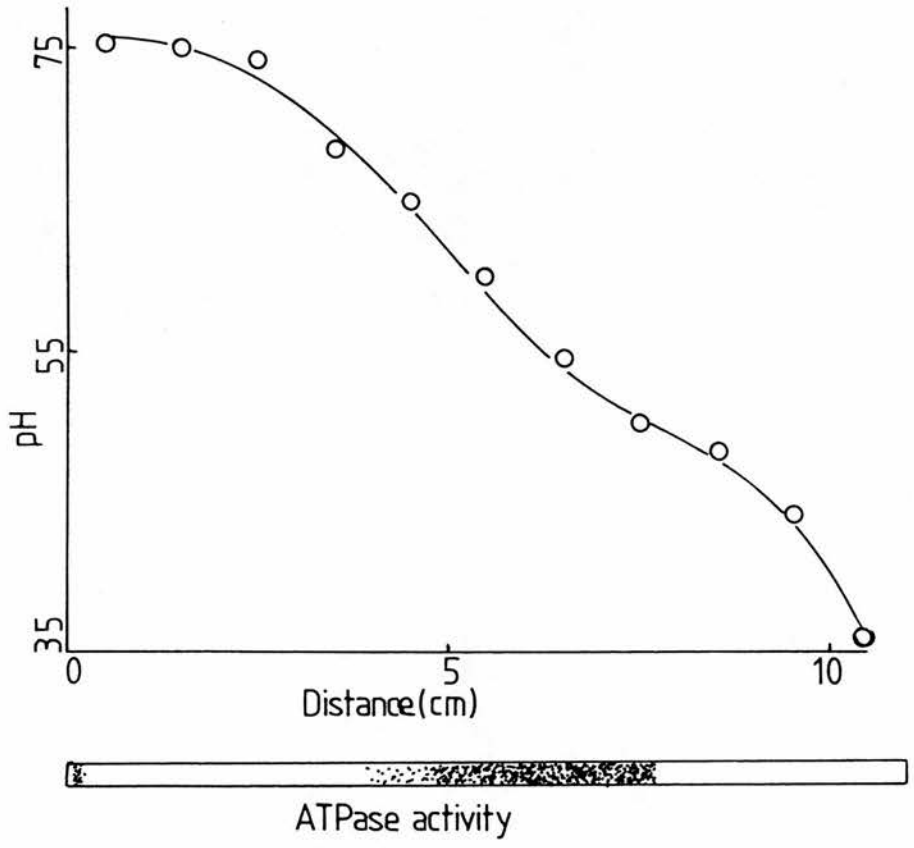


Figure 7.8. pH and ATPase activity profiles of Nondenaturing isoelectric focussing. Conditions and assays as described in Chapter 7.4.2.

Figure 7.9. Non Denaturing Two Dimensional Gel Electrophoresis of Chromaffin Granule Membranes labelled with ^{14}C-DCCD.

(a) Coomassie blue staining pattern

(b) Flourograph of gel shown in (a). See section 7.4 for details.

phoresed as described in 7.2.6, with acid solution (anode) at the bottom.

After IEF the gels were removed from the tubes and one was stained for ATPase activity (Apps and Glover, 1978) after washing out ampholines for ten minutes with distilled water. The presence of ATPase could be detected by a lead phosphate precipitate after three hours. A small band was visible at the top of the gel and was probably due to ATPase which did not enter the gel; however most of the precipitate was clustered between pIs of 4.9-6.2 with a tail from 6.2 to 6.85. This is clearly demonstrated in Figure 7.8 which shows the lead phosphate and pH distribution along the length of the tube. Two duplicate gels were electrophoresed in a second dimension as described in 7.2.7.

One gel was fixed, impregnated with sodium salicylate and auto-radiographed to reveal the DCCD-reactive proteins, whilst the other gel was subjected to immune replica using anti-serum directed against mitochondrial F_1-ATPase (chapter 2) and subsequently stained with Coomassie Blue.

7.4.3. Results

A picture of the stained gel is presented in Figure 7.9. In contrast to IEF in urea the pattern obtained without urea was highly repeatable. In the presence of 2-mercaptoethanol less protein appeared to enter the IEF tube and so the focussing pattern in Figures 9(a) and (b) and Figure 10 is for IEF in the absence of 2-mercaptoethanol. Cytochrome b_{561} focussed between pH 7.4 and pH 7.3, whilst another basic protein (pI 7.4-7.45) was clearly visible. The pI for the cytochrome is different to that observed by Apps et al. 1980 and is probably due to the absence of urea in this system giving a different protein conformation. As observed previously there is some evidence

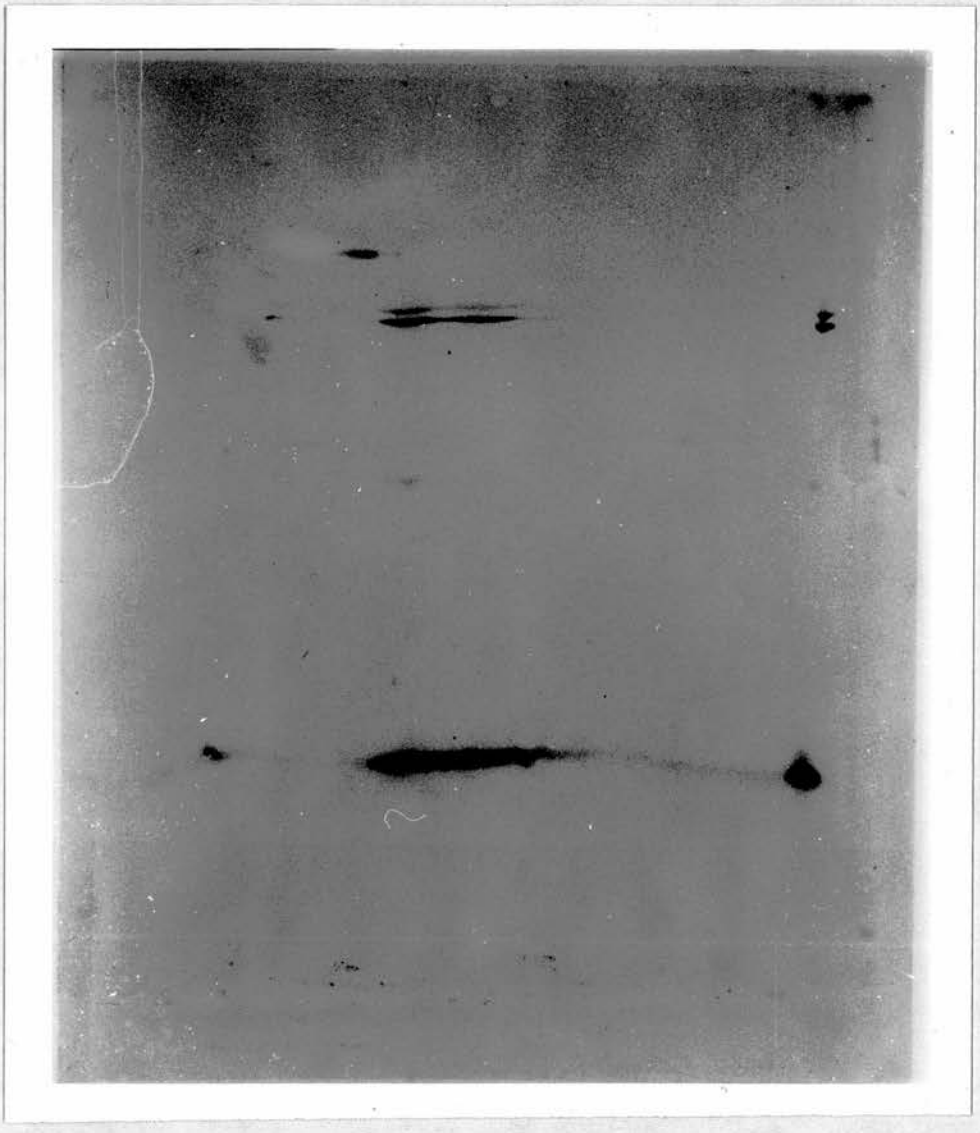


Figure 7.10. Profile of antigens to beef heart mitochondrial F_1 ATPase antibodies obtained using non denaturing two dimensional gel electrophoresis of chromaffin granule membranes

Pouring and electrophoresis of gels are described in 7.4.2. The gel was subsequently subjected to immune replica determination of F_1ATPase using antiserum directed against beef heart mitochondrial F_1ATPase.

for the cytochrome being polymeric whilst in the membrane, and the difference in isoelectric points observed here and elsewhere may be due to the protein migrating as an oligomer in non denaturing IEF. Chromogranin A, also clearly visible, focusses at a pI of 5.05-5.53, well within limits previously observed; this lends weight to postulate a random coil structure for the protein (Sen and Sharp, 1980). Other proteins are as yet undefined, but the reproducibility of the pattern may help in the identification of the many other peptides resolved by this technique.

The autoradiograph of this gel is shown in Figure 7.9(b). Of immediate note is the presence of a spot with the mobility of the DCCD-reactive protein in the second dimension, with pI = 5.5-5.85. Its focussing pattern relative to F_1 ATPase is discussed later in this chapter. Also clearly visible are three other proteins which covalently bind DCCD. One is cytochrome b_{561} which has already been discussed. The second is the 80-120 000 molecular weight protein which focusses at pI = 4.3. Its acidity may make purification of this protein possible.

Also visible is a spot which focusses near to chromogranin, (pI = 5.15) but does not have the same distribution as chromogranin A. Its pI is at the more acidic end of the pI range for chromogranin A, which is inconsistent with its being the same protein, as DCCD binding would tend to make the protein more basic.

Even if all the aspartate and glutamate residues in the DCCD-reactive protein are the free acids, rather than their amides, the pI of the denatured protein is calculated to be 6.75: since the observed pI is 5.5-5.9 the protein is probably not denatured, but part of a multisubunit complex.

Figure 7.10 shows the position of F_1 ATPase subunits of the nondenaturing IEF. The α and β subunits migrate together in three

places. One is material at the top of the tube where γ-subunit and an unidentified low molecular weight anti-F_1 reactive subunit are situated. This may well account for the narrow band of ATPase activity seen in Figure 7.8. The α and β subunits comigrate in two other places: at pI = 5.5-5.85 (where γ is also present) and at pI 6.1-6.6 (where the γ-subunit is not clearly visible). The small highly reactive subunit is also present approximately comigrating with the α and β subunits. Its exact nature, although of interest, is not yet defined.

The major point is that the α, β and γ subunits comigrate with the DCCD reactive protein, which gives clear evidence for an α β γ-DCCD-reactive protein complex, probably similar to the mitochondrial F_oF_1 ATPase complex. The small amounts of this complex which migrates on the gel (as judged by intensity of the DCCD-reactive protein spot) could be due to mitochondrial contamination, but the streak down the side of the gel does not indicate any visible mitochondrial contamination. Several doubts about the exact position of these proteins remain as one is comparing stained gels, salicylate impregnated gel autoradiographs, and the immune replica (performed whilst test gel was wet, with subsequent drying of the cellulose nitrate sheets). Distances migrated were calculated by taking known distance markers, i.e. length of gel when comparing staining pattern and immune replica, and the width of the gel when comparing the autoradiogram with the stained gel.

7.5 Summary

1. The two dimensional electrophoretic system of O'Farrell has been modified for use with chromaffin granule and mitochondrial membranes.
2. Electrophoresis of ^{14}C-DCCD labelled membranes under denaturing conditions failed to reveal the presence of the low molecular weight DCCD-reactive protein, although several labelled proteins of higher

molecular weight were apparent.

3. The two dimensional "map" of chromaffin granule proteins is similar to those obtained by others.

4. The chromaffin granule ATPase retains its activity during electrofocussing under nondenaturing conditions. In the absence of urea, the DCCD-reactive protein focusses together with α β and γ subunits of the F_1 like ATPase. This has demonstrated the presence of an ATPase of the F_oF_1 type in chromaffin granule membranes.

Chapter 8

General Discussion

8.1 Introduction

Since most of the detailed discussion of this work has been put forward in foregoing chapters, this final chapter will deal briefly with subjects not discussed previously. It will also outline some possible experiments which could clarify our present understanding of chromaffin granule bioenergetics, and the molecular structure of the ATPase.

8.2 The Chromaffin Granule DCCD-reactive Protein

The main bulk of the thesis was concerned with work related to the nature of inhibition of chromaffin granule ATPase activity by DCCD. In Chapter 3 the presence of a low molecular weight polypeptide, which was covalently modified by DCCD was demonstrated. These experiments also showed that this protein is not a mitochondrial contaminant, as it had greater mobility on SDS-polyacrylamide gels, than the corresponding mitochondrial DCCD reactive protein. The value of M_r = 14,600 derived from SDS-polyacrylamide gel electrophoresis of the chromaffin granule protein was rather large when compared to that of other DCCD-reactive proteins (Sebald et al., 1979(b)), but since the mitochondrial protein migrated more slowly, this was ascribed to an artefact of the gel system. It could be that the mitochondrial protein migrated as a dimer, whilst the granule protein was a monomer. Some arguments although circumstantial, suggests that the chromaffin granule protein has M_r = 7600 and runs as a monomer in SDS-polyacrylamide gels. The low molecular weight mitochondrial DCCD-reactive protein migrates more slowly on SDS-polyacrylamide gels; M_r = 7600 gives a 1:1 ratio of DCCD:DCCD-reactive protein, in keeping with results observed in other systems; the protein extracts into chloroform:methanol, of which the largest DCCD-reactive protein so far isolated has M_r = 8500; and the amino acid analyses give integral values if M_r = 7600 is assumed.

The amino acid analyses gave further evidence of the difference between the chromaffin granule and mitochondrial DCCD-reactive proteins. This evidence is supported by the amino terminal determination of the chromaffin granule DCCD-reactive protein which showed that the N-terminus was blocked, in contrast with that of the beef heart mitochondrial protein. Confirmation of these results requires sequence analysis of the polypeptide. Initial attempts at sequencing have been carried out by W. Sebald (personal communication) on DCCD-reactive protein purified and sent to him by the author. These have proved unsuccessful owing to the blocked N-terminus, and the resistance of the protein to degradation by trypsin, N-bromosuccinimide and cyanogen bromide. Obviously use of different proteases might yield some sequencable polypeptides, and from this a sequence may yet be derived. The sequence of this protein is of great interest as it is the only membrane protein so far isolated from a proton translocating ATPase whose physiological role is ATP hydrolysis. The position of the single tryptophan residue as well as the other data obtainable from sequence analysis may give greater insight into the structure of the proton channel of proton translocating ATPases of the F_oF_1 type.

Another experiment which should be attempted is the direct demonstration of the role of the chromaffin granule DCCD-reactive protein in forming proton-conducting channels. At present this is an area of great dispute, as workers on the E.coli protein have failed to demonstrate proton conductance with proteoliposomes incorporating the DCCD-reactive protein alone (Fillingame, 1981). This has lead to the hypothesis that all three membrane subunits a, b, and c are required to form a proton pore. However results from beef heart mitochondrial and chloroplast-DCCD-reactive proteins, incorporated into liposomes, have shown these liposomes to be conductive to protons and the conductance to be DCCD sensitive (Celis, 1980; Sigrist-Nelson and Azzi, 1980).

8.3. The Chromaffin Granule ATPase

Some time has been devoted in this thesis to a consideration of whether or not the low molecular weight, solvent-soluble DCCD-reactive protein functions as a part of an H^+ translocating ATPase complex. Initial evidence comes from inhibition studies. Both ATPase activity and proton translocation are inhibited by DCCD in chromaffin granule ghosts (Apps et al., 1980(b)), which has been shown to inhibit other ATPases by chemical modification of a low molecular weight subunit (Sebald and Hoppe, 1981). Thus in this respect this evidence favours the chromaffin granule ATPase being similar to those from mitochondria, as has been demonstrated by Apps and Schatz (1979). The fact that both the chemically modified proteins extract into organic solvent lends weight to the arguments that the ATPases are similar. The inhibition by DCCD of the chromaffin granule ATPase is potentiated by the addition of ATP to the incubation medium as is the covalent modification of the DCCD-reactive protein, suggesting that increased binding of DCCD to the protein may result in the increased inhibition of the ATPase.

Finally there is evidence from solubilisation experiments to suggest that the DCCD-reactive protein is part of an ATPase complex. In these, chromaffin granule membranes were treated with low concentrations of DCCD, solubilised using $C_{12}E_8$ or taurodeoxycholate, and the solubilised material resolved by molecular exclusion chromatography or isoelectric focussing. Comparison of the migration patterns of the DCCD-reactive protein, F_1ATPase and ATPase activity several facts emerged. Although the DCCD-reactive protein was always associated with F_1ATPase and ATPase activity, F_1ATPase did not always comigrate with the DCCD-reactive protein; in some column experiments it migrated as an inactive complex. The explanation offered in the text of separation of F_o from F_1 followed by cold inactivation of the F_1 complex is not the only one. $C_{12}E_8$ could

a so be forming mixed micelles with protein complexes in which the DCCD-reactive complex and F_1 ATPase comigrate. Using taurodeoxycholate these two complexes are separated. However there is one major argument against the latter hypothesis. The estimation of the molecular weight of the ATPase complex using both $C_{12}E_8$ and taurodeoxycholate solubilised material followed by molecular exclusion chromatography is 460000 which suggests also that the ATPase may be of an F_1F_o type. Also an F_1F_o complex separated by isoelectric focussing maintained ATPase activity. As the isoelectric focussing was carried out at room temperature an active F_1 complex could also be seen.

A counter argument to the theory of a DCCD-reactive protein-F_1 ATPase complex comes from stoichiometry calculations outlined in Chapter 5. These suggest a DCCD-reactive protein: F_1 ATPase ratio of 24-30. Although some F_1 ATPase may have been lost during the purification of the granule membrane, the figure is still rather high. Until it becomes possible to obtain a pure high molecular weight ATPase complex which contains $\alpha\beta\gamma$ and DCCD-reactive protein subunits one cannot be certain whether the DCCD-reactive protein is part of an F_oF_1 ATPase complex or part of some other ATPase which also translocates protons. At present the evidence favours an F_oF_1 ATPase complex.

8.4. The Peak II ATPase

During the column chromatography experiments, the presence of a second peak of ATPase activity was demonstrated. Initially it was thought this was due to detachment of F_1 ATPase from F_o but two lines of evidence argued against this. First the molecular weight of the PEAK II ATPase is of the order of 100000, much smaller than that of F_1 ATPase. Second the immune replica experiments demonstrated the absence of any anti-F_1 reactive proteins comigrating with the second ATPase. The presence of a second ATPase has long been suspected (Apps and Glover, 1978). The inhibition

data shows biphasic kinetics which can best be explained by more than one ATPase activity (Apps et al. 1980(b)). Two questions remain; whether the second ATPase is a contaminant and what is its function. It seems unlikely to be a contaminant as it comprises 35-40% of the total ATPase activity, is insensitive to inhibition by ouabain or EGTA or to cation activation, and is highly sensitive to vanadate and quercetin (Apps et al. Annals NY Acad. Sci. in the press). The function of the second ATPase remains more of a problem. The possibility that it could be concerned with exocytosis is quite attractive. It may also be part of the PI kinase altered by solubilisation with detergent. Finally evidence presented by Apps et al. (1980(b)) suggests that this ATPase may also act as a proton pump. This is based upon inhibition studies which show incomplete inhibition of both ATPase activities and proton translocation by DCCD and trialkyltins in chromaffin granule ghosts. More work must be done to elucidate the function of this ATPase. A simple experiment which could be done is to look at the vanadate sensitivity of the PI kinase. Also it may be possible to investigate the vanadate sensitivity of exocytosis by using chromaffin cells, which have had plasma membrane structure disrupted by high voltages, and allowing vanadate to perfuse the cell (Baker and Knight, 1978).

Assuming that this low molecular weight ATPase is indeed a component of chromaffin granules, it will be important to attempt to identify it among the 50-100 polypeptides known to be present in the chromaffin-granule membrane. Its lability may preclude convenient methods of protein purification, but a possible approach would be affinity labelling of chromaffin granule membranes, using covalently reacting ATP analogues, such as 8-azido ATP or 2',3' dialdehyde ATP, followed by exclusion chromatography or gradient centrifugation of detergent solubilised proteins. Identification of a protein or even

several proteins, which bind ATP and are of the same apparent molecular weight as the ATPase would permit the raising the antibodies, which might be of great value in characterising the ATPase activity.

In this context it should be pointed out that, although no detailed characterisation has been published, a number of reports have suggested that ATPases, which are not of the F_1F_o type, but which are involved in H^+-translocation may have low molecular weight DCCD reactive subunits. These enzymes from the plasma membranes of fungi, bear some resemblance in molecular weight and inhibitor sensitivity to the "Peak II" ATPase of chromaffin granules.

8.5. Other Effects of DCCD

An important feature of the reactions of carbodiimides is their lack of specificity in reacting with different amino acid residues, i.e. not only with the DCCD-reactive proteins of the ATPase complex, but other proteins such as cytochrome oxidase (Casey et al., 1980) or the phosphate transporter (Houstek et al., 1981). In this respect the chromaffin granule membrane is no different from that of mitochondria. Of the granule proteins identified as binding DCCD, cytochrome b_{561} is one, and a previously unidentified protein, M_r = 80-120000 is another. One of the transport activities known to be inhibited by DCCD is catecholamine translocation, and if this occurs by covalent modification of the transport protein, this may prove a possible means of identification, and a tool in purification of this enzyme.

Chapter 9

References

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nhibition of adenosine triphosphatase, 5-hydroxytryptamine transport and proton-translocation activities of resealed chromaffin-granule 'ghosts'

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1. Highly purified resealed chromaffin-granule 'ghosts' were assayed for ATPase and ATP-driven H^+-translocation and 5-hydroxytryptamine-uptake activities, and for 5-hydroxytryptamine uptake driven by an imposed transmembrane H^+-gradient. The effects of several inhibitors on these activities were studied. 2. Dicyclohexylcarbodi-imide inhibits all of these activities, but not in parallel; at low concentrations it decreases the permeability of the membrane to protons. 3. 4-Chloro-7-nitrobenzofuran (Nbf-Cl) and silicotungstate inhibit ATP-dependent activities, without effect on 5-hydroxytrypt-amine uptake driven by an imposed H^+-gradient. 4. Tributyltin chloride inhibits all of the activities. 5. Treatment of the 'ghosts' with low concentrations of urea inhibits 5-hydroxytryptamine uptake and ATP-dependent generation of a transmembrane H^+-gradient, without inhibiting ATPase activity. 6. Nbf-Cl and silicotungstate are without effect on the rate of leakage of 5-hydroxytryptamine from preloaded 'ghosts', whereas dicyclohexylcarbodi-imide and tributyltin chloride accelerate the rate of leakage. 7. Treatment of the membranes with ^{14}C-labelled Nbf-Cl labels several proteins; membranes treated with dicyclohexyl[^{14}C]carbodi-imide are labelled predominantly in a protein of low molecular weight, which may be analogous to the mitochondrial H^+-conducting proteolipid. 8. It is concluded that Nbf-Cl and silicotungstate inhibit the H^+-translocating ATPase of the granule membrane; that dicyclohexylcarbodi-imide inhibits the ATPase, and inhibits 5-hydroxytryptamine accumulation by accelerating leakage of the amine; and that the effects of tributyltin chloride are due to inhibition of the ATPase, and collapse of the transmembrane H^+-gradient through OH^--anion exchange.

ike the secretory vesicles of nerve terminals, the echolamine storage vesicles of the adrenal dulla, known as chromaffin granules, contain high centrations of catecholamines and ATP, as well roteins and other components (for a review, see kler, 1976). Accumulation of catecholamines nucleotides occurs via specific translocases, ake being driven by an ATPase (EC 3.6.1.3) in granule membrane, which electrogenically trans-ites protons to the interior of the granule, ting a transmembrane H^+-gradient (ΔpH) and nbrane potential ($\Delta\psi$) (Njus & Radda, 1978). mechanism by which transport is coupled to P hydrolysis is still unclear, although it appears both ΔpH and $\Delta\psi$ may be important in driving uptake of catecholamines (Njus & Radda, 1979; Johnson & Scarpa, 1979; Apps *et al.*, 1980), whereas uptake of ATP may be driven by $\Delta\psi$ alone (Aberer *et al.*, 1978).

The H^+-translocating ATPase of the chromaffin-granule membrane has not yet been isolated, but it bears some resemblance to the mitochondrial ATP-ase complex in its sensitivity to dicyclohexylcar-bodi-imide (Bashford *et al.*, 1976) and trialkyltin (Apps & Glover, 1978), and its failure to catalyse isotope exchange between ADP and ATP (Apps & Reid, 1977). Dichloromethane treatment of purified bovine adrenal chromaffin-granule membranes solu-bilizes an ATPase that is structurally and immuno-logically similar to mitochondrial F_1-ATPase (Apps & Schatz, 1979). We now report a study of the effects of several ATPase inhibitors on the ATPase and catecholamine-transport activities of resealed chromaffin-granule 'ghosts'. The aim of this work

bbreviations used: Hepes, 4-(2-hydroxyethyl)-1-razine-ethanesulphonate; Mes, 4-morpholine-ethane-honate; Nbf-Cl, 4-chloro-7-nitrobenzofuran.

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was to achieve further characterization of the H^+-translocating ATPase and its coupling to catecholamine transport.

Materials and methods

NN'-Dicyclohexyl[^{14}C]carbodi-imide and 4-chloro-7-nitro[U-^{14}C]benzofuran ([^{14}C]Nbf-Cl) were obtained from CEA, Gif-sur-Yvette, France. [^{14}C]-Methylamine hydrochloride and 5-hydroxy[*side chain* 2-^{14}C]tryptamine were from The Radiochemical Centre, Amersham, Bucks., U.K. Triphenyltin chloride was from Cambrian Chemicals. Trimethyltin chloride, tributyltin chloride, *NN'*-dicyclohexylcarbodi-imide and dodeca-silicotungstic acid were obtained from BDH. Ouabain, phlorizin, 4-chloro-7-nitrobenzofuran and ATP were from Sigma. Triethyltin bromide was the gift of Dr. A. P. Dawson, School of Biology, University of East Anglia, Norwich, Norfolk, U.K.

Resealed chromaffin-granule 'ghosts' were prepared by a modification of the method previously described (Phillips, 1974). Freshly prepared bovine chromaffin granules were lysed at 0°C by approx. 50-fold dilution into 10mM-Hepes/NaOH, pH 7.0; the sucrose concentration was re-adjusted to 0.3M, the membranes collected by centrifugation (30min, 23000rev./min, 4°C, in a Beckman type 35 rotor, $g_{av.}$ 41000), resuspended in 0.3M-sucrose to a concentration of approx. 2mg of protein/ml, and 5ml portions were layered on discontinuous gradients of 4.5ml of 0.4M-sucrose over 2.5ml of 0.4M-sucrose in ^2H_2O. All solutions were buffered with 10mM-Hepes/NaOH, pH 7.0. The gradients were centrifuged (30min, 40000rev./min, 4°C in a Beckman SW41 rotor, $g_{av.}$ 196000) and the 'ghosts' were collected from the interface between sucrose and sucrose/^2H_2O; the final protein concentration was 2.5–4.5mg/ml, and associated catecholamine approx. 150nmol/mg of protein. Specific ATP-hydrolytic and 5-hydroxytryptamine-uptake activities did not decline significantly on storage of the 'ghosts' for 2 weeks at −20°C; in all experiments reported, 'ghosts' had been frozen and thawed once only.

Purified chromaffin-granule membranes were prepared by a procedure published elsewhere (Apps & Schatz, 1979). Mitochondrial contamination was assessed by comparing the specific cytochrome *c* oxidase (EC 1.9.3.1) activity (Mason *et al.*, 1973) of each preparation with that of mitochondria; it was approx. 1.3% for purified membranes (Apps & Schatz, 1979) and 1.7% for resealed 'ghosts'. Bovine heart submitochondrial particles were prepared by the method of Hansen & Smith (1964). Protein was determined by the method of Bradford (1976).

ATP hydrolysis and ATP-dependent uptake of 5-hydroxy[^{14}C]tryptamine were determined in chr maffin-granule 'ghosts' (approx. 0.1mg of pr tein/ml) incubated at 37°C with 6mM-ATP, 3m $MgSO_4$, 59μM-5-hydroxy[^{14}C]tryptamine (sp. radi activity 8.5Ci/mol), 0.3M-sucrose, 10mM-Hepe NaOH, pH 7.0; 100μl samples were withdrawn intervals for determination of accumulated hydroxytryptamine by membrane filtration a counting for radioactivity (Phillips, 1974) or colo metric determination of P_i (Muszbek *et al.*, 197 Uptake of 5-hydroxytryptamine driven by an i posed H^+-gradient was assayed as described p viously (Phillips, 1978); 'ghosts' (0.2–0.4mg protein/ml) were preincubated for 15min at 37°C 0.3M-sucrose/10mM-Mes/NaOH (pH 5.6), th diluted with an equal volume of 88mM-Hepe NaOH, pH 8.8, in 0.3M-sucrose, containing 18μ 5-hydroxy[^{14}C]tryptamine (sp. radioactivity 58C mol). The final pH of the incubation medium w 8.0, and the transmembrane ΔpH, measured [^{14}C]methylamine distribution, was approx. 2 Samples (100μl) were removed at intervals, dilut in 2.5ml of 0.3M-sucrose/10mM-Hepes/NaC (pH 7.0) (0°C) and rapidly filtered through 25n cellulose nitrate filters (Sartorius; 0.45μm pore siz The fitlers were washed with two 2.5ml portions the same buffer, dried and counted for radioactiv in toluene-based scintillator.

Leakage of 5-hydroxytryptamine from 'ghosts' was assayed in 'ghosts' pre-loaded (30n at 30°C) in the incubation mixture used to meas ATP-dependent uptake of 5-hydroxytryptami These were diluted 50-fold into 0.3M-sucro 10mM-Hepes/NaOH (pH 7.0) (30°C) contain the required inhibitor. Samples (5ml) were remo at intervals and filtered, and the filters were wash dried and counted for radioactivity. A correct was made for the rate of re-accumulation 5-hydroxytryptamine after dilution, with 'gho pre-loaded in 50μM-5-hydroxy[^{12}C]tryptamine diluted into 5-hydroxy[^{14}C]tryptamine (0.14μM; radioactivity 58Ci/mol). Uptake of 5-hydroxy[^{14} tryptamine was then measured as before: concentration and specific radioactivity of hydroxy[^{14}C]tryptamine in the medium after d tion should be very close to the values occur when ^{14}C-labelled substrate was also present du the loading phase of the experiment.

ATP-dependent formation of a transmembr H^+-gradient (ΔpH) was determined by measu accumulation of [^{14}C]methylamine by the 'gho (Phillips, 1978) incubated (protein concentrat 0.5mg/ml) at 37°C in 6mM-ATP, 3mM-MgS 50μM-5-hydroxytryptamine, 40mM-KI, 2.5 [^{14}C]methylamine (sp. radioactivity 40Ci/m 30mM-Hepes/NaOH, pH 7.0. At intervals, 2(samples were removed and filtered without dilu through 13mm membrane filters, which

ounted for radioactivity, without washing or rying, in scintillator containing 33% (v/v) Triton -100. In calculating the size of the H^+-gradient, an ternal volume of 4 μl/mg of protein was assumed 'hillips & Allison, 1978).

Inhibition of 5-hydroxytryptamine uptake, H^+-anslocation and ATP hydrolysis was studied in hosts' that had been preincubated with inhibitor for min at 37°C, at a protein concentration of 5–0.8 mg of protein/ml. Assays were performed mediately after preincubation, and initial rates ere determined from plots of the time course of ch reaction, constructed with at least five points. nce most of the inhibitors used were hydrophobic d would be expected to partition into the mem-ane, relative activities were plotted against the tio of inhibitor to membrane protein. Where ibitors were added as ethanolic solutions, the al ethanol concentration did not exceed 1% (v/v), d was the same in all samples.

The effects of urea were studied by incubating osts' (0.6 mg of protein/ml) with various con-ntrations of freshly dissolved urea (BDH; Aristar ade) in 0.3 M-sucrose/25 mM-Hepes/NaOH H 7.8)/3 mM-EDTA. After 30 min at 0°C, the mbranes were centrifuged (40 min, $g_{av.}$ 98000) at C, the pellets rinsed with 1 ml of 0.3 M-sucrose/ mM-Hepes/NaOH, pH 7.0, and then resuspended he same medium.

To identify membrane proteins covalently labelled h dicyclohexylcarbodi-imide or Nbf-Cl, purified omaffin-granule membranes (2.0 mg of protein/) were incubated for 18 h at 22°C in 10 mM-pes/NaOH/0.1 mM-EDTA plus either 50 μM-di-cohexyl[^{14}C]carbodi-imide (sp. radioactivity Ci/mol) and 10 mM-ATP, or 25 μM-[^{14}C]Nbf-Cl . radioactivity 109 Ci/mol). The membranes were ited with 5 vol. of 10 mM-Hepes/NaOH, pH 7.0, l centrifuged (40 min, $g_{av.}$ 235000). They were suspended in the same buffer (2 mg of pro-n/ml), diluted with 4 vol. of acetone at 0°C, and trifuged (30 min, $g_{av.}$ 27000) in Corex tubes. ally the pellets were dissolved in 0.125 M-Tris/ l, pH 6.8, 2% (w/v) sodium dodecyl sulphate, and teins were separated by electrophoresis on 15 cm yacrylamide slab gels, in which the acrylamide centration increased exponentially from 10% at top to 15% (w/v) at the bottom (Douglas & ow, 1976). After electrophoresis, the gels were ned with Coomassie Blue R250, dried on to er, and autoradiographed with Curix RP-1 film fa-Gevaert).

ults

vities of resealed chromaffin-granule 'ghosts'

o test the effects of inhibitors on various gy-linked functions of the chromaffin-granule membrane, we assayed four different activities: (i) ATP hydrolysis, measured by the release of P_i from ATP; (ii) ATP-dependent uptake of 5-hydroxy-tryptamine; (iii) ATP-dependent H^+-translocation and establishment of ΔpH, indicated by the distribution of [^{14}C]methylamine; and (iv) uptake of 5-hydroxytryptamine driven by an imposed ΔpH, produced by a pH-jump in the absence of ATP. Assays (i) and (ii) were performed on samples of 'ghosts' taken from the same incubation mixture; the activities of preparations used in the experiments reported in the present paper, ±S.D., were respectively 116 ± 30 nmol/mg per min ($n = 6$) and 12.1 ± 1.2 nmol/mg per min ($n = 5$). This ATPase activity of resealed 'ghosts' is somewhat lower than that reported for purified membranes, assayed under different conditions (Apps & Schatz, 1979). Typical time courses are shown in Fig. 1(*a*), from which it can be seen that, under the conditions of our experiments, the initial rate of 5-hydroxytryptamine uptake was maintained for at least 5 min and that of ATP hydrolysis for even longer.

When measuring the establishment of ΔpH, we included 40 mM-KI in the incubation medium. High concentrations of permeant anion are inhibitory to 5-hydroxytryptamine accumulation (Phillips, 1978), but were necessary to maximize the ΔpH, and to enable H^+-translocation to be measured by the filtration method. In contrast with the hydrolysis of ATP and uptake of the amine, ΔpH was established very rapdily (Fig. 1*a*) and the rate of methylamine uptake [0.27 ± 0.06 nmol/mg per min ($n = 4$)], with 2.5 μM-methylamine in the medium, started to decline within 30 s. The methylamine concentration ratio (C_{in}/C_{out}) is plotted in Fig. 1(*a*); the logarithm of this is equal to the ΔpH generated.

In addition to uptake in the presence of ATP, accumulation of 5-hydroxytryptamine may be driven by a transmembrane H^+-gradient produced by a pH-jump in the absence of ATP. Fig. 1(*b*) shows a typical time course of such an experiment; uptake ceased after about 1.5 min, the initial rate of uptake [4.6 ± 1.8 nmol/mg per min ($n = 8$)] being maintained for about 30 s.

In the experiments described below, we report the effect of a variety of inhibitors on these values, initial rates being obtained from plots of the type shown in Fig. 1. Data for percentage inhibition refer to decreases of these initial rates; this includes measurements of the rate of H^+-translocation (as measured by methylamine distribution), so that the data in this case do not refer to the size of the maximal H^+-gradient achieved.

Inhibition of chromaffin granule membrane ATPase

A number of compounds were screened as inhibitors of ATP hydrolysis and ATP-dependent uptake of 5-hydroxytryptamine (Table 1). Azide,

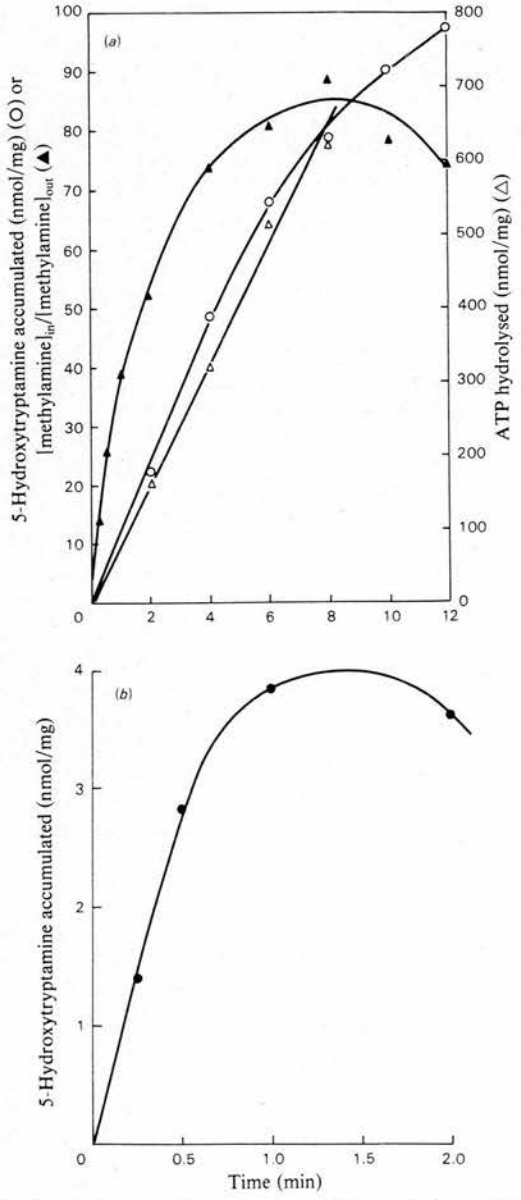

Fig. 1. *ATPase and transport activities of chromaffin-granule 'ghosts'*

'Ghosts' were assayed as described (see the Materials and methods section); time courses of (*a*) ATP hydrolysis (△), ATP-dependent 5-hydroxytryptamine uptake (○) and ATP-dependent H^+-uptake (▲) and (*b*) 5-hydroxytryptamine uptake, driven by an H^+-gradient produced by a pH-jump in the absence of ATP (●) are shown.

phlorizin, ouabain and vanadate had only slight effects, and were not studied further, whereas triethyl-, trimethyl- and triphenyl-tin were similar in action to tributyltin.

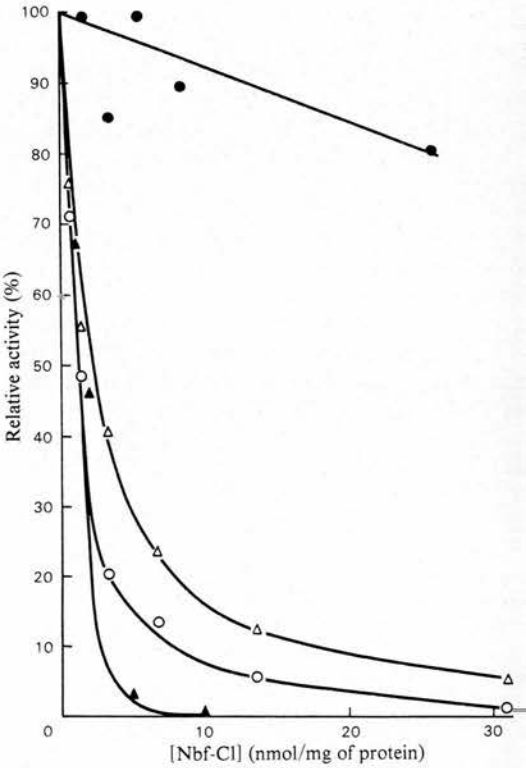

Fig. 2. *Effects of Nbf-Cl on activities of chromaf granule 'ghosts'*

'Ghosts' were preincubated with various concentrations of Nbf-Cl, and assayed for ATPase activity (△), ATP-dependent uptake of 5-hydroxytryptamine (○), ATP-dependent H^+-translocation (▲) and 5-hydroxytryptamine uptake, driven by a pH-jump (●).

Effects of Nbf-Cl

Fig. 2 shows the effects of Nbf-Cl on A' dependent and transport functions of chromaf granule 'ghosts'. All of the ATP-dependent fu tions are strongly inhibited by preincubation 5 min with low concentrations of the reagent contrast, uptake of 5-hydroxytryptamine is sensitive to Nbf-Cl if driven by a pH-jump in absence of ATP, being only 10% inhibited w ATP-dependent uptake is inhibited 95%.

Effects of dicyclohexylcarbodi-imide

The effects of dicyclohexylcarbodi-imide on c' maffin-granule 'ghosts' (Fig. 3) are complex. A ase activity is inhibited, in agreement with the re of Bashford *et al.* (1976). ATP-dependent uptak 5-hydroxytryptamine appears more sensitive inhibition by dicyclohexylcarbodi-imide than is hydrolysis, being 90% inhibited under condit

Table 1. *Inhibition of ATPase and ATP-dependent 5-hydroxytryptamine-uptake activities of resealed chromaffin-granule 'ghosts'*

'Ghosts' were assayed as described in the Materials and methods section, with the inclusion of the inhibitors in the final incubation at the concentrations shown. Percentage activities are expressed relative to controls without inhibitor, but with ethanol where appropriate.

Inhibitor	Concentration (mM)	ATPase	5-Hydroxytryptamine uptake
Azide	3.5	100	68
Dicyclohexylcarbodi-imide	0.10	42	12
Nbf-Cl	0.01	23	13
Ouabain	1.0	98	90
Phlorizin	2.0	96	100
Tributyltin chloride	0.01	31	6
Triethyltin chloride	0.01	29	17
Trimethytin chloride	0.01	38	27
Triphenyltin chloride	0.01	38	15
Sodium orthovanadate	0.01	87	86

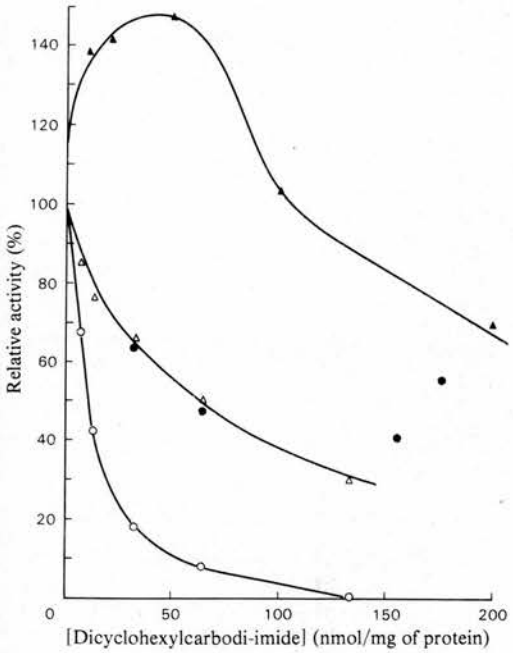

Fig. 3. *Effects of dicyclohexylcarbodi-imide on activities of chromaffin-granule 'ghosts'*
For explanation of symbols see the legend to Fig. 2.

hat produce only 50% inhibition of the ATPase; his result is in contrast with that of Bashford *et al.* 1976), who found similar inhibition of both activities. Significantly, uptake driven by an imposed H^+-gradient is also inhibited (see also Schuldiner *et l.*, 1978). However, low concentrations of dicyclohexylcarbodi-imide stimulate the initial rate of [^{14}C]methylamine accumulation, and only at inhibitor concentrations above 100 nmol/mg of protein is the rate of establishment of the H^+-gradient inhibited.

Effects of alkyltins

Of the various alkyltin derivatives found to inhibit chromaffin-granule membrane ATPase and transport activities (Table 1), tributyltin was selected for study in detail: its effects are shown in Fig. 4, and are in some ways comparable with those of dicyclohexylcarbodi-imide. ATP hydrolysis, ATP-dependent uptake of 5-hydroxytryptamine and ATP-dependent H^+-translocation are inhibited, though not in parallel, and transport driven by a pH-jump is also inhibited.

Urea and silicotungstate

Treatment of submitochondrial particles with urea or silicotungstate has been used to selectively remove the F_1-component of the H^+-translocating ATPase from the membrane (Schatz *et al.*, 1967; Racker *et al.*, 1969), and we investigated the effects of similar treatment of chromaffin-granule 'ghosts'.

Chromaffin-granule membrane ATPase was found to be fairly resistant to urea, inactivation by 2 M-urea being insignificant (Fig. 5*a*); however, this concentration of urea produced over 50% inactivation of 5-hydroxytryptamine uptake and of methylamine accumulation. Silicotungstate is a potent inhibitor of the ATPase and of the ATP-dependent functions 5-hydroxytryptamine uptake and H^+-translocation, whereas uptake driven by an imposed H^+-gradient is insensitive to this inhibitor (Fig. 5*b*).

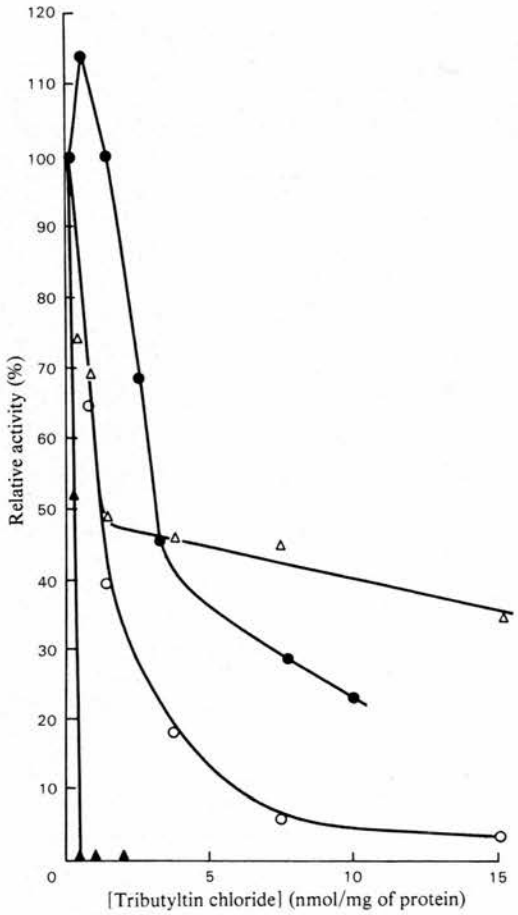

Fig. 4. *Effects of tributyltin chloride on activities of chromaffin-granule 'ghosts'*
For explanation of symbols see the legend to Fig. 2.

Leakage of 5-hydroxytryptamine from pre-loaded 'ghosts'

If chromaffin-granule 'ghosts' are pre-loaded by incubating with 5-hydroxytryptamine in the presence of ATP, then diluted into iso-osmotic buffer so that the concentrations of 5-hydroxytryptamine and ATP are much less than the K_m values of the carrier and the ATPase, the concentration gradient is dissipated by efflux. The effects on leakage of various inhibitors, included in the dilution medium, are shown in Fig. 6. The experiments were performed at 30°C, to decrease the rate of 5-hydroxytryptamine efflux in the absence of inhibitors, and each trace is corrected for re-accumulation occurring after dilution (see the Materials and methods section). For comparison, the effects of two uncouplers, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and K^+ plus nigericin, were also investigated.

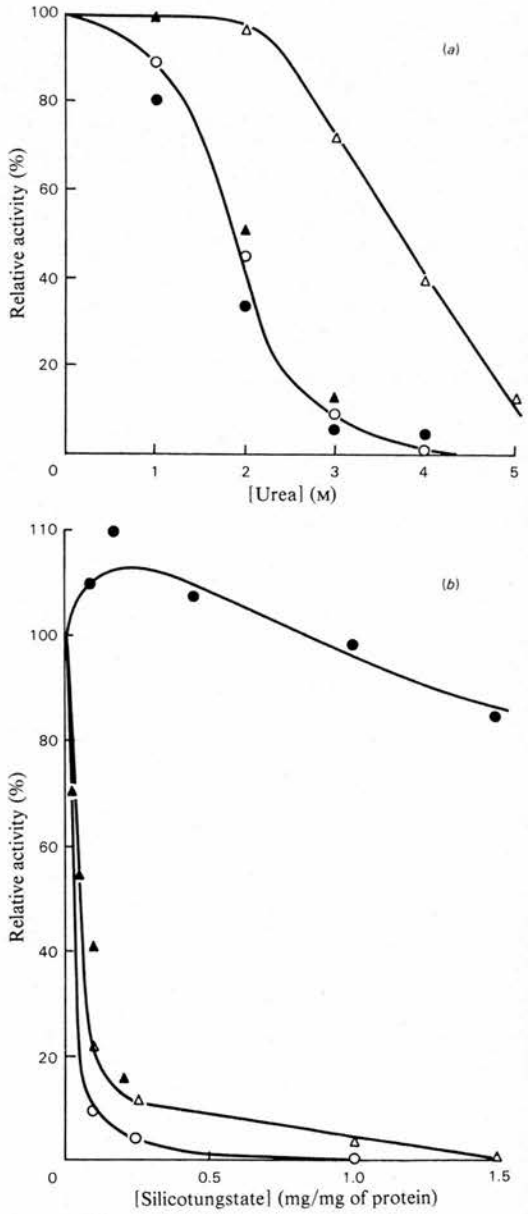

Fig. 5. *Effects of (a) urea and (b) silicotungstate on activities of chromaffin-granule 'ghosts'*
For explanation of symbols see the legend to Fig. 2. The silicotungstate concentration shown is that used in preincubation of the 'ghosts'; it was diluted 10-fold in the assay media. The urea concentration shown is that used in preincubation of the 'ghosts', which were washed by centrifugation before assay.

Covalent modification of membrane proteins

Two of the inhibitors used, Nbf-Cl and di cyclohexylcarbodi-imide, probably exert their effect through covalent modification of proteins in th

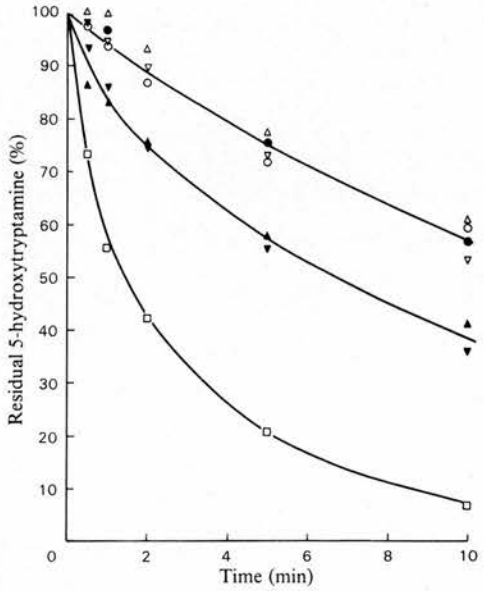

Fig. 6. *Effects of inhibitors on leakage of 5-hydroxytryptamine from preloaded 'ghosts'*

After loading with 5-hydroxytryptamine, 'ghosts' (0.7 mg of protein/ml) were diluted 50-fold into iso-osmotic buffered sucrose, containing the following additions: ○, none; ●, Nbf-Cl (5 μM); △, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (10 μM); ▽, silicotungstate (200 μg/ml); ▲, dicylohexylcarbodi-imide (50 μM); ▼, tributyltin chloride (2 μM); □, nigericin (50 ng/ml) + K_2SO_4 (20 mM).

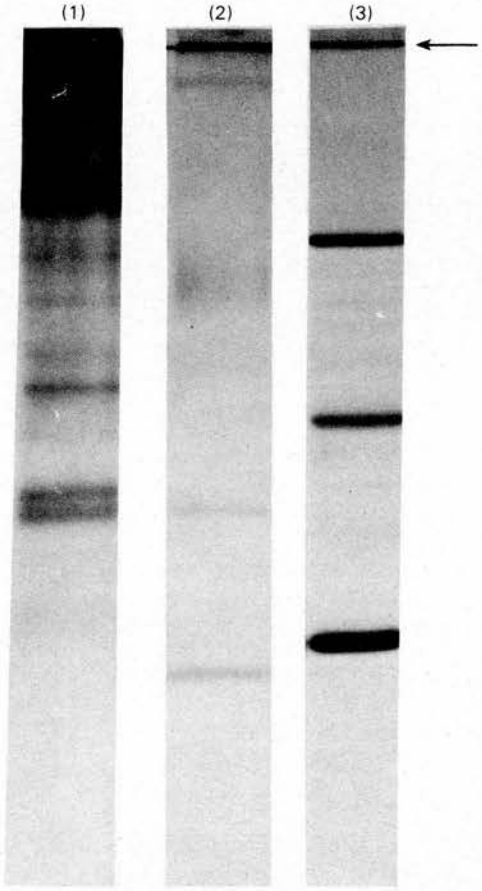

Fig. 7. *Reaction of [^{14}C]Nbf-Cl and dicyclohexyl-[^{14}C]carbodi-imide with purified chromaffin-granule membranes*

Autoradiograph of ^{14}C-labelled proteins after separation by dodecyl sulphate/polyacrylamide-gel electrophoresis. (1), Chromaffin-granule membranes labelled with [^{14}C]Nbf-Cl; (2), chromaffin-granule membranes and (3) submitochondrial particles, labelled with dicyclohexyl[^{14}C]carbodi-imide. The arrow indicates the top of the separating gel.

chromaffin-granule membrane. Both are non-specific reagents, likely to modify more than one protein. The number of proteins reacting was investigated by preincubation of purified chromaffin-granule membranes with dicyclohexyl[^{14}C]carbodi-imide or [^{14}C]Nbf-Cl followed by separation of membrane proteins by electrophoresis in the presence of dodecyl sulphate, and identification of radioactive proteins by autoradiography of the dried gel. The results are shown in Fig. 7. Labelling with dicyclohexyl[^{14}C]carbodi-imide was carried out in the presence of 10 mM-ATP, as this has been found to increase the incorporation of radioactivity (R. Sutton, unpublished work). For comparison, samples of bovine heart submitochondrial particles were also labelled, under the same conditions.

Discussion

The chromaffin-granule ATPase

How similar are the H^+-translocating ATPases of mitochondria and chromaffin granules? This question is prompted by the observation (Apps & Glover, 1978; Apps & Schatz, 1979) that dichloromethane treatment of purified chromaffin-granule membranes solubilizes a protein that is in many ways similar to mitochondrial F_1-ATPase. The inhibitors dicyclohexylcarbodi-imide, Nbf-Cl and tributyltin, found in the present work to inhibit chromaffin-granule membrane ATPase, also inhibit mitochondrial ATPase, but since all three are potentially non-specific, this does not necessarily reflect structural similarity between the two enzymes. Indeed, some or all of the inhibitors studied have been reported to inhibit the ATPases of synaptic vesicles (Toll & Howard, 1978), gastric mucosa (Sachs *et al.*, 1976) and the plasma membranes of *Neurospora crassa* (Bowman *et al.*, 1978) and *Schizosaccharomyces pombe* (Delhez *et*

al., 1977), all of which may be H^+-translocating enzymes. Silicotungstate inhibits the ATPase activity of submitochondrial particles, high concentrations causing dissociation of F_1-ATPase from the membrane (Racker *et al.*, 1969), but its effects on chromaffin-granule 'ghosts' seem to be due to inhibition of the ATPase, without its dissociation; dodecyl sulphate/polyacrylamide-gel electrophoresis of silicotungstate-treated chromaffin-granule membranes reveals no depletion of the α- and β-subunits of the ATPase, and the membranes are still agglutinated by antibodies against mitochondrial F_1-ATPase (D. K. Apps & J. G. Pryde, unpublished work). Attempts to reconstitute the ATPase activity of these membranes by addition of mitochondrial F_1-ATPase have been unsuccessful. Urea (3 M) fails to remove or inactivate the ATPase, suggesting that it may be more firmly attached to the membrane than is the mitochondrial enzyme.

In mitochondria, bacteria and chloroplasts, dicyclohexylcarbodi-imide inhibits ATP-dependent H^+-translocation by covalent modification of the proteolipid that constitutes the proton-conducting channel (Cattell *et al.*, 1971; Fillingame, 1976; Sigrist-Nelson *et al.*, 1978); alkyltins may also act by binding to the membrane segment of the enzyme, rather than to the F_1-ATPase itself (Cain & Griffiths, 1977). Does chromaffin-granule ATPase contain a similar proton-conducting proteolipid subunit? Two pieces of evidence suggest that it does. First, treatment of chromaffin-granule membranes with dicyclohexyl[^{14}C]carbodi-imide reveals a major covalently-reacting protein of low molecular weight, with greater mobility in dodecyl sulphate/polyacrylamide-gel electrophoresis than the mitochondrial proteolipid. Reaction of dicyclohexyl-[^{14}C]carbodi-imide with a mixture of mitochondrial and chromaffin-granule membranes labelled two electrophoretically distinct proteins; the difference in mobility observed (Fig. 7) is therefore not an artefact of the gel system, and the protein labelled in chromaffin granules cannot be a mitochondrial contaminant (R. Sutton, unpublished work). Secondly, low concentrations of dicyclohexylcarbodi-imide increase the rate of establishment of the ATP-generated transmembrane H^+-gradient; this could be due to the inhibitor preferentially blocking H^+-channels with non-functional or detached ATPase, and thus decreasing the H^+-permeability of the membrane without much inhibition of the H^+-pump: only at high dicyclohexylcarbodi-imide concentrations, when active H^+-translocation is extensively inhibited, is development of the H^+-gradient affected. The stimulatory effects of low concentrations of dicyclohexylcarbodi-imide on photophosphorylation in chloroplasts has been explained similarly (McCarty & Racker, 1967).

Even high concentrations of dicyclohexylcarbodi-imide or tributyltin fail to totally inhibit the chromaffin-granule membrane ATPase, suggesting either that a fraction of the enzyme is dislocated from the H^+-conducting channel, and hence is unaffected by its blockage, or that the membrane contains more than one ATPase.

Catecholamine translocase

Catecholamine uptake by chromaffin granules is catalysed by a specific permease, as indicated by its stereospecificity and inhibitor sensitivity (Phillips, 1974). A variety of biogenic amines is transported, including 5-hydroxytryptamine as well as catecholamines (Kanner *et al.*, 1979).

It is apparent from Figs. 3 and 4 that dicyclohexylcarbodi-imide and tributyltin have effects on 5-hydroxytryptamine uptake that are not due solely to inhibition of the ATPase, since uptake produced by an imposed H^+-gradient is also inhibited. Possible explanations for this include direct inhibition of the translocase, dissipation of the transmembrane H^+-gradient, or acceleration of catecholamine leakage. Measurements of the rate of 5-hydroxytryptamine leakage (Fig. 6) indicate that both dicyclohexylcarbodi-imide and tributyltin increase the rate of leakage above that observed in controls, or in Nbf-Cl- or silicotungstate-treated 'ghosts'.

The mechanism by which 5-hydroxytryptamine leaks from 'ghosts' is unknown. Evidence has been presented that catecholamine uptake occurs by electrogenic exchange of protonated amine with protons (Johnson & Scarpa, 1979); under the conditions used in leakage experiments it is possible that 5-hydroxytryptamine leaves, via the translocase, by reversal of this process, in which case agents that increase the H^+-permeability of the membrane would promote leakage. However, uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, although they inhibit ATP-driven uptake of catecholamines by chromaffin granule 'ghosts' (Bashford *et al.*, 1975), do not accelerate leakage. Since uncouplers of this type catalyse electrogenic H^+-uniport, this suggests that electroneutral dissipation of the H^+-gradient may be required; it is therefore particularly noteworthy that a combination of K^+ and nigericin, which permits K^+/H^+ exchange, does indeed promote rapid leakage of 5-hydroxytryptamine from the 'ghosts' although nigericin alone has only slight effects on the rate of leakage. It seems likely, therefore, that passage of unprotonated amine through the membrane may take place. This presumably also occurs in H^+-gradient-driven uptake of catecholamines by liposomes (Nichols & Deamer, 1976). It should be noted, however, that it is not possible to exclude

specific internal effect of the K^+ that is taken up by the 'ghosts' in the presence of nigericin. The alkyl tins have been shown to catalyse rapid OH^--anion antiport in membranes (Selwyn *et al.*, 1970) and tributyltin might therefore dissipate the transmembrane H^+-gradient electroneutrally, by transporting anions from the interior of the 'ghosts'. It is not clear whether high concentrations of anions are accumulated during loading with 5-hydroxytryptamine, since the only anions known to be present are SO_4^{2-}, Hepes ATP^{4-}; but tributyltin strongly inhibited the generation of the transmembrane H^+-gradient, as measured by methylamine distribution, since 40mM-KI was present in these experiments. We tentatively conclude that the effects of this reagent on 5-hydroxytryptamine uptake by the 'ghosts' are due to its effects on the H^+-gradient.

In contrast, dicyclohexylcarbodi-imide accelerates leakage, although apparently decreasing the H^+-permeability of the membranes; this is confirmed by ΔpH measurements. This high leakage rate, the mechanism of which is at present obscure, presumably accounts for the effects of dicyclohexylcarbodi-imide on 5-hydroxytryptamine uptake seen in Fig. 3.

Although it is difficult to explain the effects of some of these inhibitors in detail, it is clear that the data are fully consistent with the idea that the ATPase and the catecholamine translocase are separate entities. Thus, Nbf-Cl and silicotungstate abolish ATPase activity, ATP-dependent H^+-translocation and ATP-dependent uptake of 5-hydroxytryptamine, without affecting the translocase. In contrast, low concentrations of urea inhibit translocation without affecting the ATPase, apparently by increasing the H^+-permeability of the membrane. This duality of action is strong evidence in favour of the indirect coupling of transport to ATPase activity via the H^+-gradient. Recently, Maron *et al.* (1979) have reported the differential solubilization of the translocase and the ATPase by cholate treatment of membranes.

The insensitivity of the translocase to Nbf-Cl is interesting, in view of the demonstration (Fig. 7) that this reagent reacts covalently with many proteins in the chromaffin-granule membrane. Although dicyclohexylcarbodi-imide appears to react predominantly with a single membrane protein, as judged by electrophoresis, it has a complicated effect on the membrane, as shown by its acceleration of amine leakage (Fig. 6). Both dicyclohexylcarbodi-imide and tributyltin are also noteworthy for their incomplete inhibition of the ATPase, suggesting either that some of the membrane ATPase is not coupled to H^+-translocation, or that there is an additional inhibitor-insensitive ATPase present. This clearly merits further investigation, as does the question of the mechanism of 5-hydroxytryptamine leakage.

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ISOLATION OF A DCCD-BINDING PROTEIN FROM BOVINE CHROMAFFIN-GRANULE MEMBRANES

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1. Introduction

Chromaffin granules, the secretory granules of the adrenal medulla, contain a membrane-bound, proton-translocating ATPase which, like the ATPase of mitochondria, chloroplasts and bacterial plasma-membranes, is inhibited by DCCD and by the alkyl tins [1,2]. The transmembrane proton-gradient and membrane potential established by this enzyme are utilized in the accumulation of catecholamines by the granules [3,4]. Treatment of purified bovine chromaffin-granule membranes with dichloromethane solubilizes an ATPase which is very similar, but not identical, to mitochondrial F_1-ATPase [5]. Since the solubilized chromaffin-granule enzyme is not inhibited by DCCD or alkyl tin, it is probably derived from a complex with a membrane-bound segment, containing a proton-conducting channel which is blocked by these inhibitors. This idea is supported by kinetic studies and by reconstitution experiments [2,6,7]; furthermore, treatment of chromaffin-granule membranes with [^{14}C]DCCD, followed by SDS electrophoretic separation of membrane components and autoradiography of the electrophoretograms, reveals the labelling of a low M_r polypeptide, with a greater electrophoretic mobility than the DCCD-binding protein of mitochondria [2]. We now report isolation of this protein, and determination of its amino acid content.

Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase (EC 3.6.1.3); DCCD, *N*,*N*′-dicyclohexyl carbodi-imide; Hepes, *N*-2-hydroxyethyl-*N*′-2-ethane sulphonic acid; SDS, sodium dodecyl sulphate; M_r, relative molecular mass

2. Materials and methods

Chromaffin granule membranes of high purity were prepared as in [5]. ATPase activity was measured at 30°C by following NADH oxidation in a coupled assay system containing 1.0 mM ATP, 10 mM $MgSO_4$, 0.5 mM phosphoenol pyruvate, 0.2 mM NADH, 5.5 units lactate dehydrogenase/ml and 4 units pyruvate kinase/ml, 50 mM KCl and 50 mM Hepes–KOH (pH 7.4). Since the NADH oxidase activity of the membranes was ~10 nmol . min^{-1} . mg $protein^{-1}$, no correction was necessary. Protein concentrations were estimated by the method in [8]. SDS–Polyacrylamide gel electrophoresis and autoradiography were performed as in [2].

Determination of N-terminal amino acids was attempted after treatment of the purified protein with dansyl chloride in SDS [9]. The dansylated protein was hydrolysed (6 M HCl, 20 h, 105°C), vacuum dried and redissolved in 50% aqueous pyridine. Dansyl amino acids were separated by chromatography on polyamide plates, the solvents being 1.5% formic acid in the first dimension, toluene–acetic acid (9:1) in the second dimension (run perpendicular to the first), and butyl acetate–methanol–acetic acid (30:20:1) for the third separation, run in the same direction as the second. Amino acid analysis was performed on ~10 nmol protein hydrolysed in 6 M HCl at 105°C for 40 h or 90 h. Amino acid separation was on a Locarte analyser, using a 3-buffer step elution system [10]. Cysteine was measured at cysteic acid after performic acid oxidation [11]. *N*,*N*′-Dicyclohexyl-[^{14}C]carbodi-imide (spec. act. 50 μCi/ml) was supplied by CEA (Gif-sur-Yvette).

3. Results and discussion

3.1. *Inhibition of chromaffin granule ATPase by DCCD*

Even high concentrations of DCCD (up to 150 nmol/mg protein) produce only partial inhibition of the ATPase [1,2], but it was noted that incorporation of label into a low M_r protein was greatly stimulated when ATP was included during the preincubations of the membranes with [^{14}C]DCCD. Fig.1 shows that ATP also increases the rate and extent of inactivation of ATPase by DCCD.

3.2. *Extraction of the DCCD-binding protein*

The DCCD-binding proteins from many sources have been purified after extraction into chloroform–methanol mixtures [12] or into butanol [13]. The protein from chromaffin-granules is extracted by chloroform–methanol (2:1) (though not butanol) but the high lipid content of the chromaffin-granule membrane hinders subsequent isolation of the protein from the chloroform–methanol extract. This problem was overcome by first extracting lipids from the membrane by treatment with acetone–ethanol (1:1, v/v) then extracting the DCCD-binding protein from

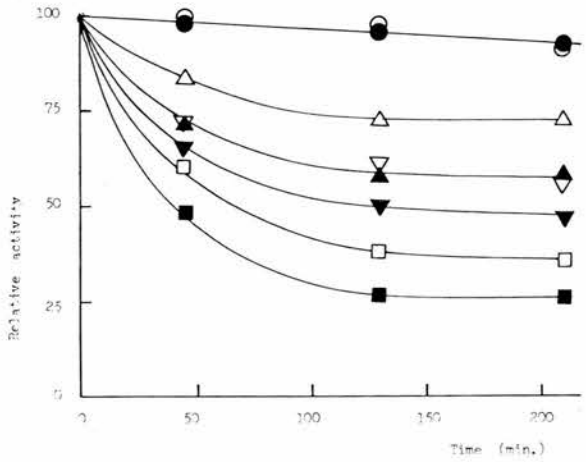

Fig.1. Effect of ATP on inhibition of chromaffin-granule membrane ATPase activity by DCCD. Chromaffin-granule membranes (1.0 mg protein/ml, in 0.1 M Hepes–NaOH (pH 7.0)) were incubated at 25°C with various concentrations of DCCD, and samples removed at intervals for assay. The initial ATPase activity was 420 nmol . min^{-1} . mg protein^{-1} at 30°C. Solid symbols, 10 mM ATP present during incubation; open symbols, no ATP. DCCD concentrations were: 0 (○,●), 10 (△,▲), 25 (▽,▼) and 100 (□,■) nmol/mg protein.

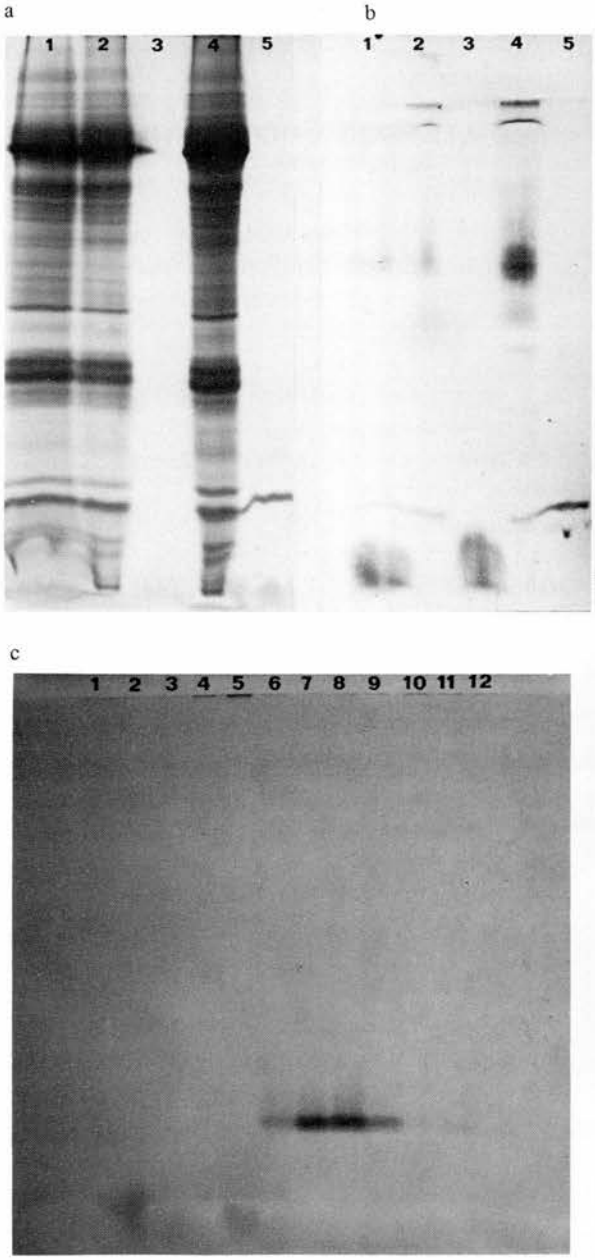

Fig.2. SDS–polyacrylamide gel electrophoresis. (a) Extracts of chromaffin-granule membranes which had been treated with [^{14}C]DCCD; gel stained for protein with Kenacid blue: (1) labelled membranes; (2) residue from acetone–ethanol extraction; (3) supernatant from acetone–ethanol extraction; (4) residue from chloroform–methanol extraction; (5) supernatant from chloroform–methanol extraction. (b) Autoradiograph of a gel similar to that in (a). (c) Successive fractions of elute from the Sephadex LH-20 column used to purify the DCCD-binding protein. Gel stained with Kenacid blue.

the insoluble residue into chloroform–methanol. A polyacrylamide gel of fractions from each stage of this double solvent extraction (fig.2a) and the autoradiograph of a similar gel (fig.2b) show that the DCCD-binding protein is completely insoluble in acetone–ethanol, and is extracted into chloroform–methanol. The protein so extracted appears to be almost pure by the criterion of polyacrylamide gel electrophoresis (fig.2a (5)), although it contains some low M_r impurities which are probably lipids. Further purification was achieved by chromatography on Sephadex LH-20, yielding the pure material shown in fig.2c.

3.3. *Purification of the chromaffin-granule DCCD-binding protein*

The following procedure was adopted for purification of the protein. When performed on membranes preincubated with [^{14}C]DCCD, it yielded labelled protein; alternatively, the protein could be labelled after isolation, by incubation with [^{14}C]DCCD in chloroform–methanol solution.

Pure chromaffin-granule membranes (200 mg protein, suspended in 24 ml 10 mM Hepes–NaOH (pH 7.0)) were treated with 600 ml acetone–ethanol (1:1). After 5 min at 25°C the mixture was centrifuged (10 min, 15 000 rev./min, 4°C, in a Beckman JA20 rotor, g_{av} = 18 000), the supernatant discarded, and the pellet resuspended in 24 ml water. Chloroform–methanol (2:1) 600 ml, was then added, and the mixture stirred slowly for 5 h at 4°C, then centrifuged as before. The pellet was discarded, and the supernatant rotary-evaporated almost to dryness. The extracted material was redissolved in 10 ml chloroform–methanol (2:1 containing 4% (v/v) water) and applied to the top of a 30 cm × 5.1 cm^2 column of Sephadex LH-20 pre-equilibrated with chloroform. The protein was eluted at room temperature with a 500 ml linear gradient of chloroform to chloroform–methanol (1:1), and 35 ml fractions collected. Individual fractions were concentrated by rotary evaporation and the protein contents examined by SDS–polyacrylamide gel electrophoresis. Those fractions containing DCCD-binding protein of the highest purity were pooled, rotary-evaporated to dryness, redissolved in 2 ml chloroform–methanol (2:1) and washed 3 times with 0.5 ml water. The purified protein was readily soluble in chloroform–methanol (2:1, containing 4% water), and was stored as a solution at 4°C. The entire procedure yielded 30–50 nmol pure protein.

3.4. *N-Terminal amino acid determination*

Attempts to determine the identity of the N-terminus of the protein have been unsuccessful, the only dansyl amino acids in hydrolysates of the dansyl chloride-treated protein being N^6-dansyl lysine and *O*-dansyl tyrosine. This suggests that the amino-terminus is blocked, a result confirmed by the failure of the Edman degradation procedure to yield phenylthiohydantoin derivatives of amino acids (W. Sebald, personal communication). The amino-termini of DCCD-binding proteins from *Saccharomyces cerevisiae* mitochondria and spinach chloroplasts are also blocked; in each case the N-terminus is formylmethionine, a result of the intra-organellar synthesis of the protein [14]. However, this reason would not apply to the protein from chromaffin-granules, and it is noteworthy that the DCCD-binding proteins of beef heart, mouse liver and *Neurospora crassa* mitochondria, all of which are probably cytoplasmically synthesized, have unblocked N-termini [14,15].

Table 1
Amino acid content of DCCD-binding protein from chromaffin-granule membranes

Amino acid	Analysis 1	Analysis 2	Integral figure
Asp	3.25	2.99	3
Thr	2.22	2.20	2
Ser	9.69	9.56	10
Glu	4.44	3.85	4
Pro	3.32	2.98	3
Gly	7.85	8.32	8
Ala	9.24	9.14	9
Cys	0.11	–	0
Val	4.29	6.20	6
Met	3.17	2.19	3
Ile	3.45	5.03	5
Leu	5.81	7.17	7
Tyr	1.16	1.07	1
Phe	2.81	3.15	3
His	0.09	0.06	0
Lys	2.00	2.00	2
Arg	2.13	2.17	2
Try	–	–	–

Each analysis is the average of results derived from 40 h and 90 h hydrolyses, with the following exceptions: threonine and serine were extrapolated to zero hydrolysis time, 90 h hydrolysis values were used for leucine, isoleucine and valine, and the 40 h hydrolysis value for methionine. The methionine value is in agreement with that for methionine sulphone in the 40 h hydrolysate of performic acid-oxidized protein; this analysis was also used for cysteine

3.5. *Amino acid analysis*

The amino acid content of the protein, determined from analysis of total acid hydrolysates and expressed in mol/2 mol lysine, is shown in table 1. The integral value of 2 for lysine was chosen as this gave a M_r-value in agreement with that suggested by the mobility of the protein on SDS–polyacrylamide gels; it also results in approximately integral values for the other amino acids (table 1). The total number of amino acids is 68, compared to 75 in the beef mitochondrial protein [14]: this may account for the higher electrophoretic mobility of the protein from chromaffin granules [2]. The proportion of hydrophilic amino acids is somewhat higher than that found in other DCCD-binding proteins [14], although the content of asparagine and glutamine is unknown, and the 10 serine residues make a major contribution. This apparently high content of serine could derive from contamination of the protein with phosphatidyl serine, although no dansyl serine was found in hydrolysates of dansyl chloride-treated protein.

4. Conclusion

The protein purified from chromaffin granules is very similar in its properties to the low M_r, DCCD-binding subunits of proton-translocating ATPase from the membranes of mitochondria, chloroplasts and bacteria. Nonetheless, the differences in amino acid content and electrophoretic mobility between the DCCD-binding proteins of chromaffin granules and mitochondria are great enough to exclude the possibility that the former is of mitochondrial origin. A number of lines of evidence suggest that it is a subunit of the proton-translocating ATPase complex of chromaffin granule membranes:

(i) Increasing inhibition of ATPase activity by DCCD correlates with increasing incorporation of label from [^{14}C]DCCD into the protein; both inhibition and labelling are potentiated by ATP;

(ii) Treatment of resealed granule 'ghosts' with low concentrations of DCCD appears to decrease the proton-permeability of the membrane, suggesting that the inhibitor blocks a proton channel [2];

(iii) The DCCD-binding protein co-elutes with ATPase activity, when chromaffin granule membrane components are solubilized with a non-denaturing detergent, and separated by exclusion chromatography (unpublished).

It therefore appears that the structural similarity found for the F_1 portions of chromaffin-granule and mitochondrial ATPases [5] may extend to the membrane segments of these enzymes.

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